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semisynthesis of cytochrome c analogues

p.j. boon

SEMISYNTHESIS
OF
CYTOCHROME C ANALOGUES

SEMISYNTHESIS
OF
CYTOCHROME C ANALOGUES

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de
wiskunde en natuurwetenschappen
aan de Katholieke Universiteit te Nijmegen
op gezag van de Rector Magnificus
Prof.dr. P.G.A.B. Wijdeveld
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op maandag 15 juni 1981
des namiddags te 4 uur

door

PETRUS JOHANNES BOON

geboren te Beverwijk

Promotor : Prof.dr R.J.F. Nivard
Co-referent: dr G.I. Tesser

voor mijn vader en moeder,

voor Annemiek, Brigitta,
Cindy en Elles

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INTRODUCTION

CHAPTER I

INTRODUCTION

1.1. General introduction

The synthesis of proteins having well defined, biological activities is one of the major goals of peptide chemistry. Great progress in methodology has been made in the last two decades, as is exemplified by the syntheses of complex peptides such as human insulin (Sieber *et al*, 1974, 1977) and human big gastrin (Choudhury *et al*, 1976; Wünsch *et al*, 1977) *via* solution methods, and of fully active, bovine pancreatic trypsin inhibitor (Tan and Kaiser, 1976) and ovine β-lipotropin (Yamashiro and Li, 1978) *via* the solid phase technique.

Nevertheless, the synthesis of polypeptides containing over 100 amino acid residues has only rarely been accomplished with success. Pancreatic ribonuclease A, containing 124 amino acids, and constructed from 29 segments which were mainly coupled by the azide method (Yajima *et al*, 1980) seems to be the only synthetic protein with equal properties as the natural enzym. On the other hand, efforts to prepare ribonuclease T_1 (Finn and Hofmann, 1976), cytochrome c (Borin *et al*, 1976), lysozyme (Galpin *et al*, 1976) and Δ^5-3-ketosteroid isomerase (Kisfaludy *et al*, 1976) have been terminated prematurely or have not yet been completed.

In syntheses by segment condensations in solution, the practical limitations to the preparation of peptides longer than approximately 60 amino acid residues are due to the generally poor solubility of large protected peptides, to the decrease in coupling rates with increasing segment size, and to the increased risk of irreversible, intramolecular side reactions or *pseudo* first-order interactions of the activated carboxyl component with the solvent, including racemisation. Additionally complete removal of protecting groups under conditions mild enough to avoid product decomposition becomes increasingly troublesome with growing peptide chain length. All of these constraints are sequence dependent and appear to be hardly predictable.

In repetitive methods of synthesis in solution, the difficulty to

distinguish long peptides differing in only one or a slight number of residues poses a limit of 20-30 amino acid residues to the attainable sequence length. Similarly in the solid phase method of synthesis the formation of microheterogeneous products which can no longer be fractionated rigorously, sets this limit at 30-40 amino acid residues.

Attempts to surmount these limitations will inevitably be extremely laborious (for a more detailed discussion see Gross and Meienhofer, 1979) and can only be attempted by specialized laboratories.

In view of the obstacles in the methodology of total synthesis of proteins the alternative strategy of protein semisynthesis is currently being studied more frequently. In this strategy, readily available peptides and proteins serve as the starting materials for the preparations of analogues of the natural product.

1.2. Semisynthesis in peptide chemistry

The term semisynthesis was applied originally by Smyth (1975) to 'the combination of two components, one being a fragment of a natural substance, the other a product formed by chemical synthesis'. This wide application of the word is now replaced by a more restricted definition. Here it is assumed that only products originating from two or more fragments of different origin and *coupled through a peptide bond*, be the result of a semisynthesis. The designation '*partial synthesis*' for such a process is favoured by Sheppard (1980).

Two general approaches for semisyntheses can be discerned. In the first a natural peptide or protein is used as a source of peptide fragments, which are liberated by chemical procedures or by proteolytic enzymes. A characteristic feature of this approach, which is the most generally applicable form of protein semisynthesis, is the necessity of maximal side chain protection. The method is best exemplified by the extensive studies of Offord and coworkers (Rees and Offord, 1976; Offord *et al*, 1976) using lysozyme as the model protein: methods were developed for suitable protection of tryptic peptides, for the selective liberation of the C-terminal carboxyl groups in the fragments by making use of the esterase activity of trypsin, and for the subsequent coupling of the fragments and the final deprotection of the products. These studies remain the main effort in this type of strategy. A *priori*, this

approach is not expected to yield significant advantages over the total synthetic approach, since in addition to the encountered difficulties in the selective protection of the various side chain functional groups, the problems of the coupling of large protected fragments and the subsequent removal of protecting groups are met here as well, even under less favourable conditions.

In the second approach strategies are studies which allow a minimum manipulation of the natural peptide or protein: single amino acid residues or peptide sequences are selectively removed, so that the number of re-assembly steps is minimized. A characteristic of this approach is that protection of side chains of the natural substance is required usually only for amino groups. The approach lacks flexibility, due to the small number of methods suitable for limited fragmentation of a protein. The Edman degradation for removal of N-terminal amino acid residues has been employed widely. Examples include soybean trypsin inhibitor (Kowalski and Laskowski, 1976), phospholipase A_2 (Slotboom and de Haas, 1975) and many studies on insulin (*e.g.* Geiger *et al*, 1978). The cyanogen bromide-induced cleavage at Met residues, which occur infrequently in proteins, is another suitable method for the preparation of large fragments. Similarly, chemical procedures for cleavage after Trp (Savage and Fontana, 1977) have found some application (*e.g.* Wang *et al*, 1978).

The selective activation of the C-terminal carboxyl group of an acyl component of natural origin, in the presence of side chain carboxyl groups, presents the major obstacle in the design of a semisynthetic strategy. Currently this problem has been solved satisfactorily only in special cases. The use of proteolytic enzymes in the reverse manner, *viz* for the synthesis rather than the hydrolysis of peptide bonds, provides a potentially powerful method. Addition of organic co-solvents to displace the equilibrium towards synthesis has been studied in a number of model systems (*e.g.* Morihara and Oka, 1981) and has already found application in an enzymatic semisynthesis of human insulin from porcine insulin (Morihara *et al*, 1979), of ribonuclease (Homandberg and Laskowski, 1979) and of cytochrome c (Homandberg *et al*, 1980; Westerhuis *et al*, 1981). From a thermodynamical viewpoint the enzymatic condensation seems to be limited to cases in which either the acyl

or the amine component can be applied in large excess. Apart from systems in which the tertiary structure aids in bringing together the appropriate C- and N-termini, this condition will be difficult to meet when large protein fragments are involved. In this context, the recent observations by Yagisawa (1981) seems to be very valuable.

It was shown that model substrates for trypsin could be converted into Boc- or Z-protected hydrazides by the enzyme. These enzymatic reactions proceed almost quantitatively, even in aqueous solution. This provides potentially, a general method of converting tryptic peptides into intermediates which can be coupled subsequently to amine components by the azide method.

Another special solution to the problem of selective carboxyl activation is provided by the C-terminal homoserine lactone formation upon CNBr cleavage at Met residues. This forms the basis for the strategy developed in this thesis (Chapter II).

1.2. Cytochrome c

1.2.1. Function of cytochrome c

Cytochrome c forms part of the eukaryotic, mitochondrial, respiratory chain. This chain is composed of a series of electron carrier proteins capable of undergoing reversible oxidation and reduction. The system is located in the inner mitochondrial membrane. The main function of the chain (Figure 1) is to link the energy yielding cycles of catabolism with the synthesis of adenosine triphosphate (ATP), the main source of chemical energy for living systems.

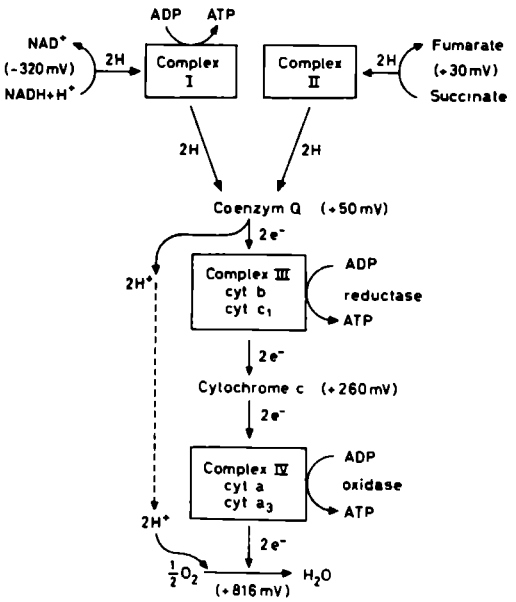


Fig 1 The mitochondrial respiratory chain.

Reduced nicotinamide adenine dinucleotide (NADH) and succinate, produced by catabolic pathways, donate reducing equivalents to the respiratory chain. These are then passed along the chain, resulting in the final reduction of oxygen to water. The passage of electrons through the electron carrier proteins, down a redox potential gradient, releases energy. This energy is trapped partially by the synthesis of ATP at three sites of the chain.

Cytochrome c is an essential link in this transport chain. It functions as a peripheral protein which carries electrons from a membrane-immobilized reductase (cytochrome $b+c_1$ Complex III) to an equally immobilized oxidase (the cytochrome $a+a_3$ Complex IV).

1.2.2. Properties of cytochrome c

Cytochrome c was first isolated in 1930 from Delft yeast by Keilin. Due to its easy extraction from the electron transfer chain, its ease of purification, and its small size, cytochrome c is, along with hemoglobin, the most studied protein. More than 80 sequences of cytochromes c from eukaryotic species, and over 35 sequences of prokaryotic cytochromes c are know to date (Ferguson-Miller *et al*, 1979). The physical properties have been thoroughly investigated (Margoliash and Schejter, 1966).

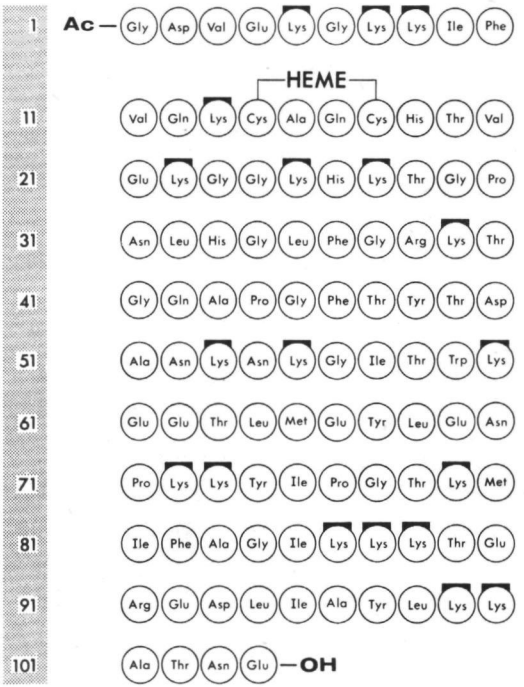


Fig 2 Amino acid sequence of horse heart cytochrome c

Eukaryotic cytochrome c is a monoheme protein, containing 103-111 amino acids, with a reduction potential of 250-260 mV. The protein occurs in two stable oxidation states, the paramagnetic ferricytochrome c (s=1/2)

and the diamagnetic ferrocytochrome c. Ferricytochrome c is stable over the pH range 4 to 8 at 25°, and to temperatures up to 60° at pH 6. Ferrocytochrome c is even more stable (Butt and Keilin, 1962).

The amino acid sequence of horse heart cytochrome c, the protein studied in this work, is shown in Figure 2. The prosthetic group in cytochrome c is protoporphyrin, in which the vinyl side chains are linked to two cysteine residues of the protein chain *via* thioether bonds. This arrangement is referred to as heme (Figure 3).

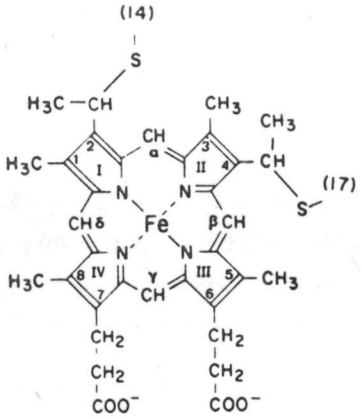
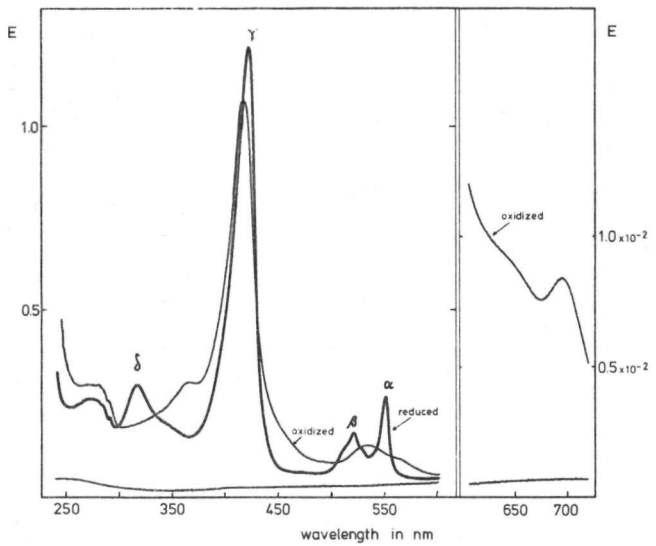


Fig 3 Heme c, the prosthetic group in cytochrome c. The four pyrrole rings are numbered I to IV, the peripheral β-positions 1 to 8, and the four meso-positions α to δ. The attachment to the protein is by two thioether bonds to Cys^{14} and Cys^{17}.



The UV and visible spectra of the oxidized and reduced forms of cytochrome c are given in Figure 4 together with the convential designation of the main absorption bands of the reduced compound.

Fig 4 UV-visible absorption spectra of oxidized and reduced horse heart cytochrome c

1.2.3. Structure of cytochrome c

Three dimensional structures derived from X-ray studies have been reported for horse and bonito ferricytochrome c (Dickerson *et al*, 1971; Matsuura *et al*, 1979),bonito ferrocytochrome c (Ashida *et al*, 1973; Tanaka *et al*, 1975), and for tuna ferri- and ferrocytochrome c (Swanson *et al*, 1977; Takano *et al*, 1977). In addition to the structures of these mitochondrial cytochromes c, structures are know for cytochrome c_2 of the photosynthetic bacterium *rhodospirillum rubrum* (Salemme *et al*, 1973), for cytochrome c_{550} of the denitrifying bacterium *Paracoccus denitrificans* (Timkovich and Dickerson, 1976), for cytochrome c_{551} from *Pseudomonas aeruginosa* (Almassy and Dickerson, 1978) and for cytochrome c_{555} from *Chlorobium thiosulfatophilum* (Korszun and Salemme, 1978).

The most remarkable result of these studies is that all these cytochromes show a common core structure of folding, notwithstanding the fundamental differences in the electron transport chains in which they function (Dickerson *et al*, 1976; Salemme, 1977).

A schematic representation of tuna cytochrome c is reproduced in Figure 5. The heme group is held rigidly within the protein framework by four bonds: two covalent thioether bonds connecting the side chains of the rings I en II to Cys^{14} and Cys^{17}, and two axial, coordinate bonds of iron with two strong field ligands, *viz* the His^{18} imidazole nitrogen and the Met^{80} thioether sulfur. The protein moiety contains 5 α-helical segments, an N-terminal helix at the top of the protein running from residues 1 to 12, a long C-terminal α-helical stretch includ-

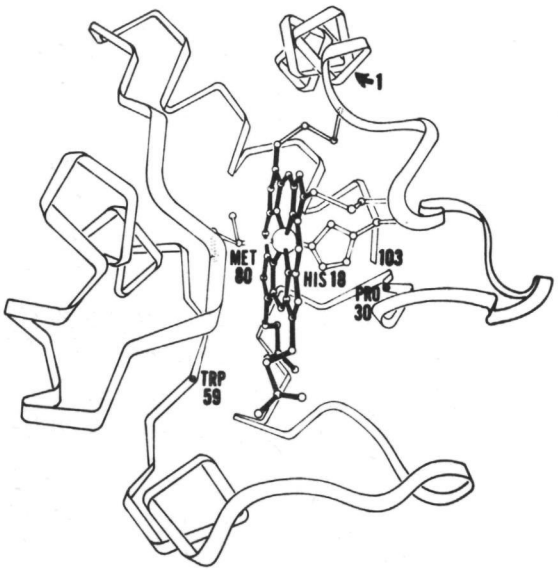


Fig 5 Ribbon diagram showing the main chain folding of the protein moiety around the heme group in mitochondrial cytochrome c (tuna heart; after Salemme, 1977).

ing residues 89-101, running perpendicular to the heme at the backside of the molecule, and three smaller α-helical regions containing the residues 50-55, 60-68 and 70-75. The α-helical structures at the termini as well as that in the region 60-68 appear to be invariant among all cytochromes c. Besides the α-helical structures several type II 3_{10} bends, a hydrogen bonding pattern in a local arrangement of three residues, appear to be conserved in the secondary structure of the cytochromes (Timkovich, 1979).

The protein is folded around the heme in such a way that only the pyrrole ring II and the edge between the rings II and III are exposed to the medium. The remainder of the heme is packed with hydrophobic side chain residues of the protein sequence. Furthermore, it is very characteristic that the two propionic acid residues of the heme are shielded from the medium. The rear propionic acid residue is completely buried within the molecule. This is realized by an extensive pattern of hydrogen bonds provided by the side chains of Trp^{59}, Tyr^{48}, Arg^{38} and Asn^{52}. Similarly, the front propionic acid residue, lying just beneath the surface of the molecule, is hydrogen bonded to Thr^{78} and Thr/Ser^{49}.

Aromatic side chains* occur in clusters, *viz* Phe^{10} and Tyr^{97} at the upper right side at the back of the molecule, the so-called 'right channel'; Tyr^{74}, Tyr^{67} and Trp^{59} at the left side, the 'left channel', and Phe^{46} and Tyr^{48} at the bottom of the molecule. The invariant Phe^{82} is within the heme crevice.

The constancy of the structural characteristics in the known cytochrome c structures, mentioned above, has been discussed at length by Dickerson and Timkovich (1975), Salemme (1977), Dickerson *et al* (1976) and Timkovich (1979). The major implication is that a unified mechanism of electron transfer must exist at some level.

Two early proposals for the mechanism of electron transfer to and from cytochrome c involve specified amino acid residues:

- in the Dickerson-Winfield mechanism for reduction (Takano *et al*, 1973; Dickerson, 1974) the electron was supposed to be conducted from the

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*The position of the aromatic groups within the molecule can be viewed from the diagrams reproduced in the inside back cover.

outside of the protein to the heme iron *via* the aromatic side chains stacked in the left channel, *viz* Tyr^{74}, Tyr^{67} and Trp^{59}

- in the mechanism proposed by Salemme *et al* (1973a,b) for *Rhodospirillum rubrum* cytochrome c_2, the redox process was supposed to be an essentially reversible process involving direct interaction between the heme with the prosthetic groups of the oxidase or the reductase; the electron transfer should be facilitated by the perturbation of a hydrogen bonding system involving the residues Tyr^{67}, Thr^{78} and Met^{80} (tuna numbering) upon complexation with the oxidoreductases.

Both mechanisms have been abandoned, mainly because the crucial amino acid residues did not appear to be invariant (Dickerson and Timkovich, 1976). Extensive chemical modification studies (reviewed by Ferguson-Miller *et al*, 1979) have revealed that a ring of positive charges at the surface of the molecule is involved in the binding of cytochrome c to various redox partners (cf Section 6.4). The binding domain on cytochrome c, as defined by these charges, appears to be at the upper front of the heme crevice, roughly centered around Phe^{82} and encompassing lysine residues 8, 13, 27, 72, 73, 86 and 87. This suggests strongly that the exposed edge of the heme is the probable site of electron transfer. Following this suggestion Salemme (1976, 1977) constructed a hypothetical complex of cytochrome c with cytochrome b_5, by optimizing complementary charge interactions between the positive charges on the front side of cytochrome c and the negative charges on the front side of cytochrome b_5. Similarly, a hypothetical electron transfer complex between cytochrome c and cytochrome c peroxidase has been described recently, in detail, by Poulos and Kraut (1980).

The complexes involve hydrogen bond interactions (2.7 to 3.0 Å) between lysines 13, 27, 72, 86 and 87 on cytochrome c and specified acidic residues on the surface of the other protein. In both cases the hemes were found to be nearly parallel, albeit displaced from a common plane by 6.8 Å in the complex with cytochrome c peroxidase. The complexes appeared to fit remarkably well. Gaps did not exist at the intermolecular interfaces, so that the solvent is effectively excluded. In the complex with peroxidase, Ile^{81}, whose side chain is exposed on the surface of cytochrome c, was found to be in the contact area, whilst Phe^{82} of cytochrome c and His^{180} of the peroxidase were found to line up along

the path between the hemes. Poulos and Kraut (1980) suggest that the electron transfer may proceed through a supramolecular conduction orbital composed of π-orbitals from both hemes, with additional contributions of protein side chain groups.

1.4. Outline of the thesis

The development of a method for the semisynthesis of horse cytochrome c analogues was the main goal of the work described here. The strategy that was envisaged at the onset of the studies (Figure 6, route B) relied on two premises:

1. that the properties of Hse^{65}-cytochrome c, the product of the conformationally guided formation of the Hse^{65}-Glu^{66} bond in a 1:1 complex of the complementary fragments 1-65 and 66-104 (Corradin and Harbury, 1974), would not be different from those of the native cytochrome c in any major respect
2. that the methylsulphonylethyloxycarbonyl (Msc) group (Tesser and Balvert-Geers, 1975), would be applicable as a reversible protecting group of the side chain amino functions of cytochrome c.

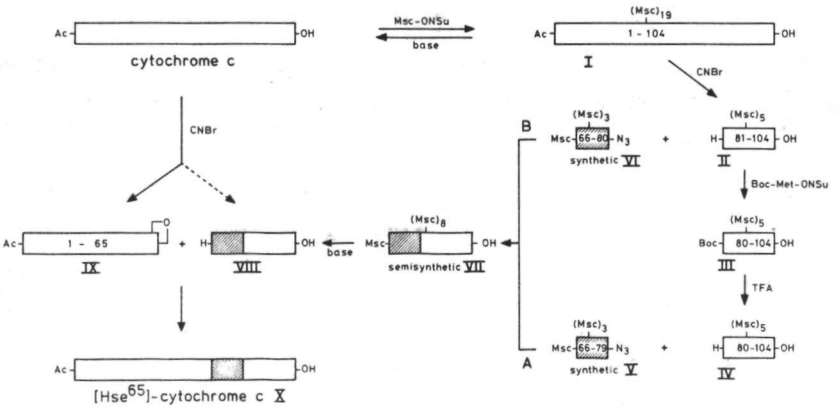


Fig 6 Strategy for the semisynthesis of Hse^{65}-cytochrome from three fragments.

The optimization of the synthesis of Hse^{65}-cytochrome c from two fragments, 1-65 and 66-104, both isolated from the native protein, and the

characterization of the protein analogue are the subject of Chapter II.

The reversible protection of amino groups in cytochrome c and the isolation of the selectively N^{ϵ}-Msc-protected cytochrome c fragment 81-104 (II) are described in Chapter III.

The synthesis by solution methods of the Msc-protected peptide derivatives V and VI, which correspond to the cytochrome c sequences 66-79 and 66-80, respectively, is reported in Chapter IV.

In Chapter V the preparation of the semisynthetic sequence 66-104 (VIII), *via* either route A and B, and its subsequent homoserine lactone mediated coupling with the heme fragment 1-65 to give Hse^{65}-cytochrome c, are described. Chemical, physical and biological comparisons with the native protein are included.

The methods developed in Chapters II-V were used to prepare seven analogues of cytochrome c, acetylated at positions 72, 73 and 79. The effects of the removal of charge at these positions on the interaction of cytochrome c with purified cytochrome c oxidase are discussed in Chapter VI.

The preparation of two analogues of cytochrome c in which either Tyr^{74} or Tyr^{67} is replaced by leucine, is described in Chapter VII. The solution structure of these analogues, as determined by 360 MHz NMR spectroscopy, is compared with that of Hse^{65}-cytochrome c. The functional significance of the two conservative residues will be discussed.

CHAPTER II

CONFORMATION DIRECTED SYNTHESIS OF HSE^{65}-CYTOCHROME C

CONFORMATION DIRECTED SYNTHESIS OF HSE^{65}-CYTOCHROME C

Re-formation of a peptide bond between the residues 65 and 66 was chosen as the ultimate step in the strategy for the synthesis of cytochrome c analogues, modified in the sequence 66-80. Therefore, the present investigations began with a study of the recombination of the 1-65 and 66-104 moieties, obtained by CNBr-treatment of the native enzyme. A precise knowledge of the effects of the Met^{65}-Hse^{65} exchange, which accompanies this procedure, was felt to be a prerequisite for the assessment of effects from changes caused by purposive substitutions in the sequence 66-80.

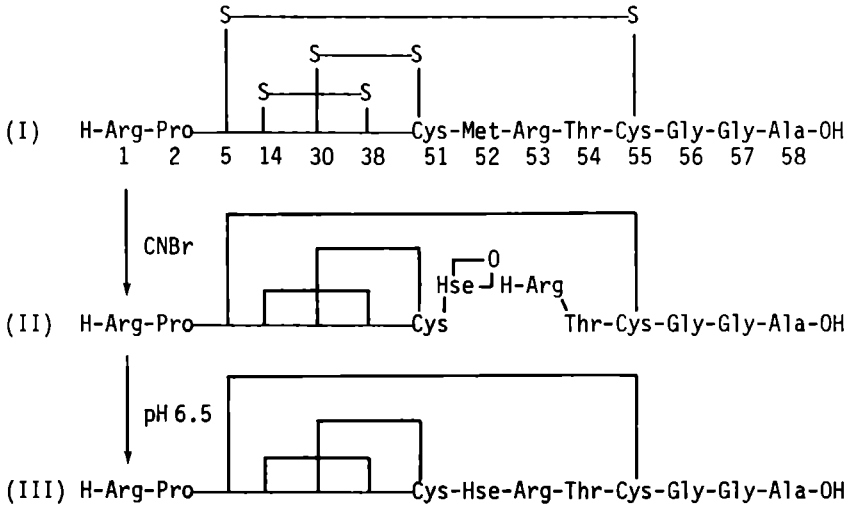
Immediately prior to these investigations, the reconstitution of horse heart cytochrome c in this way was reported in a short communication by Corradin and Harbury (1974). We repeated their work with the aim to establish optimal conditions for the resynthesis and to determine more accurately the enzymatic properties of the Hse^{65}-cytochrome c, thus obtained.

2.1. Conformation directed synthesis of proteins

Dyckes, Creighton and Sheppard (1974) were the first to describe the spontaneous re-formation of a peptide chain, broken by a cyanogen bromide. Splitting of the Met^{52}-Arg^{53} bond by basic pancreatic trypsin inhibitor (BPTI), whose structure is partially shown in Figure 1, with CNBr produced the lactone II, in which the peptide chain has been cleaved, but the peptide 53-58 is still bonded covalently by a disulfide bridge to the complementary part 1-52. When a solution of this *seco*-lactone (II) was allowed to stand at neutral pH for some days re-formation of the 52-53 peptide bond was shown to take place to an extent of up to 95%. The half-life time for the resynthesis was about 30 hours (Dyckes *et al*, 1974, 1978).

The formation of |Hse^{52}|-BPTI (III) by nucleophilic attack of the amino function of Arg^{53} on the lactone ring of Hse^{52} in II must be due to the proximity of the reacting groups, since a lactone ring is only very weakly activated. In BPTI the original Met^{52}-residue is located

Figure 1

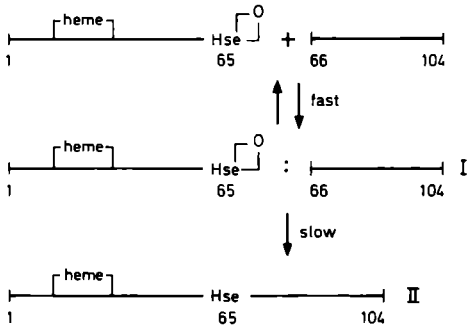


near the center of an α-helical segment which is tightly bound by disulfide bonds to the rest of the protein. Probably, this tertiary structure is largely retained in the chain-cleaved lactone II. This results in a high concentration of paired reacting groups, which may even reduce the rate of competitive hydrolysis of the lactone by a partial exclusion of water from the reaction center. Consequently, the reaction was substantially slowed down in denaturing media such as 6M guanidine-HCl or dimethylsulfoxide, while removal of Arg^{53} from II by amino peptidase gave a derivative which was inert to chain resynthesis (Dyckes *et al*, 1978). It was further shown (Dyckes *et al*, 1977) that aminolysis of the *seco*-lactone II by other peptides in denaturing media only proceeded at a rate comparable with the intramolecular reaction, when high concentrations of such nucleophiles (1M, in the presence of 7.5 mM of the lactone) were applied.

Shortly after this discovery by Dyckes, the genuine *inter*molecular condensation of two genuinely separated fragments of horse heart cytochrome c, *viz* the heme peptide 1-65 lactone and the C-terminal peptide 66-104, to an intact protein was reported by Corradin and Harbury (1974). Previously (Corradin and Harbury, 1971), the re-appearance of cytochrome c properties on *mixing* of the same fragments had been ascribed to com-

plexation: interaction of the reduced heme peptide 1-65 with the complementary non-heme peptide 66-104 in 0.1M sodium acetate buffer pH 4.7 should give a reduced complex, that upon oxidation was shown to possess the same UV-visible spectrum and circular dichroism spectra as the native ferri-protein and to be active in the succinate oxidase system. These properties were only found when the reduced complex was permitted to stand in solution for 24 hours or more. The time interval was explained as that needed for a slow recovery of the native structure, supposed to be of little consequence for the stability of the reduced complex, but of major influence on the properties of the oxidized complex. It was not until the discovery by Dyckes *et al* that it was realized that this slow process concerned the spontaneous re-formation of the 65-66 peptide bond (Figure 2).

Figure 2

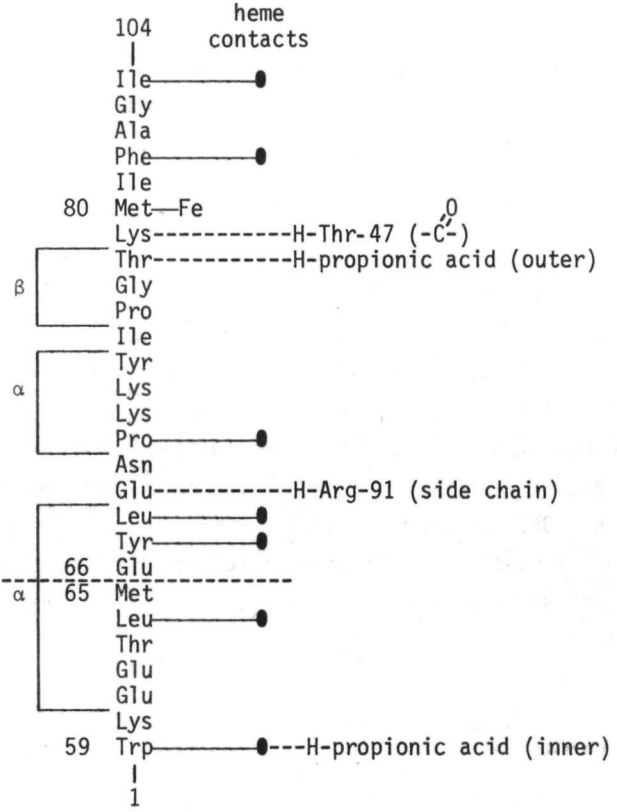


The initial complex formation was shown to be a first order process, for which the rate constant ranges from 13 s^{-1} at pH 4 to 2.4 s^{-1} at pH 8, as determined by stopped flow measurements (Marks and Harbury, 1975). The subsequent aminolysis of the homoserine^{65}-lactone by the α-amino function of Glu^{66} proceeds then in a slow reaction with an estimated half life time of approximately 6-8 hours.

The tertiary structure of the reduced complex I must closely resemble that of the native protein. A number of features of the complex may account for the rigidity of the region around the cleavage point are shown schemetically in Figure 3.

The hydrogen bonds of Trp^{59} and Thr^{78} with the two propionic acid residues of the heme moiety, the packing of hydrophobic side chains against

Figure 3



the heme and the α-helical structure of the sequence 61-69, may explain the correct positoning of the loose ends of the interacting fragments, and contribute to the selective aminolysis. These interactions alone, however, are apparently not sufficient. In solution the complex of the oxidized 1-65 lactone with peptide 66-104 appears to have a rather open conformation with an exposed heme groups (Wilgus *et al*, 1978). This does not allow smooth amide formation. X-ray differentiation of crystals of the ferri- and ferro-form of cytochrome c do not show structural differences (Mandel *et al*, 1977).

However, various physical and chemical properties of cytochrome c measured in solution indicate a more rigid structure for the reduced molecule (Dickerson and Timkovich, 1976). The better attainment of the

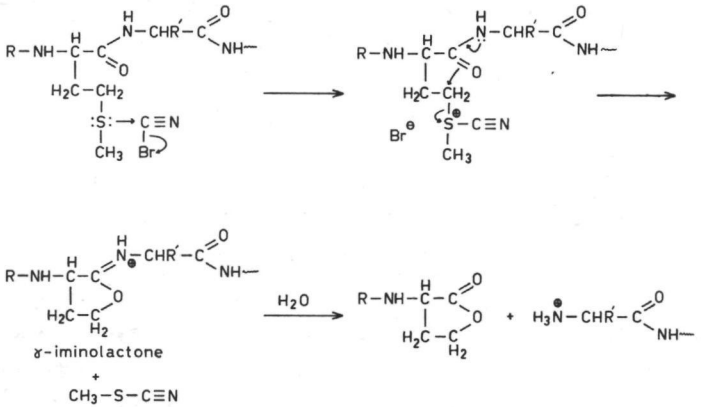
native conformation in the reduced complex can partly be ascribed to the firmer coordination of Met^{80} to the heme iron (Harbury *et al*, 1965). Moreover, removal of the formal positive charge from iron in the ferri-complex may facilitate the approach of the initially protonated α-amino function of Glu^{66}.

2.2. Cyanogen bromide cleavage

The reaction of cyanogen bromide with peptides containing methionine was shown by Gross and Witkop (1961) to lead to cleavage of the amide bond following the methionine residue. The method was subsequently adapted by the same authors to cleave ribonuclease (Gross and Witkop, 1962). Since then the unique specificity of this cyanogen bromide treatment in acidic media has been generally recognized, and the cleavage procedure has become a standard method in sequence determinations of proteins (Gross, 1967; Needleman, 1975).

The reaction is based on an electrophilic attack by cyanogen bromide on the thioether sulfur atom, which produces a cyanosulphonium bromide as a first intermediate (Figure 4).

Figure 4



The intermediate looses methyl thiocyanate with simultaneous formation of a γ-iminolactone. The cyclization step and the subsequent hydrolysis of the resulting immonium ion, giving a C-terminal homoserine lactone and a free amine, proceed readily at room temperature. This condition determines the attractiveness of the method as compared to

an earlier procedure for the cleavage of methionyl peptides, which consisted of two discrete steps, *viz* alkylation with iodoacetamide at 35-40°, followed by decomposition of the resulting S-carboxamidomethyl methionine sulphonium salt at elevated temperature (95°; Gross, 1967).

The yields of cleavage are generally high (70-100%) when a large excess of cyanogen bromide is used. However, cyanogen bromide treatment sometimes leads to conversion of methionine into homoserine without cleavage of the peptide chain (Narita and Titani, 1968; Cunningham *et al*, 1968; Schröder *et al*, 1969). In these instances the methionine residue involved was always followed by a threonine or a serine residue.

Furthermore it has been reported that N-terminal methionine residues are sometimes incompletely liberated during protein sequence analysis studies (*e.g.* Chang *et al*, 1976).

Another side reaction has been detected during cyanogen bromide treatment of proteins: up to 20-25% chain cleavage after tryptophan can occur when a large excess of the reagent is used (Braunitzer and Aschauer, 1975; Wootton *et al*, 1974).

2.2.1. *Preparation and properties of cyanogen bromide fragments of cytochrome c*

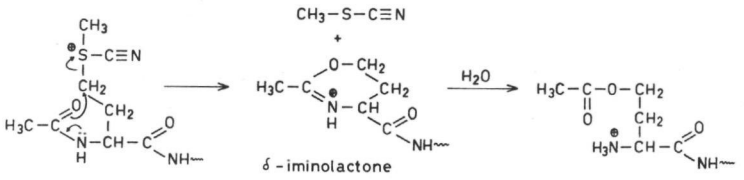
The cleavage of cytochrome c with CNBr was first studied by Black and Leaf (1965), who used a 30-fold molar excess of the reagent in 0.1N HCl as the solvent. Three fragments were isolated by repeated gel filtration on Sephadex G-50 and G-75 with 0.2N acetic acid as the eluent. Amino acid analyses gave values which agreed reasonably well with those of the sequences 66-80, 81-104 and 1-80. Methionine could not be detected, however, in the heme containing fragment 1-80. The results suggested a preponderant cleavage at Met^{80}, although this was not quantified.

McGowan and Stellwagen (1970) performed the same reaction in 63% aqueous formic acid with a larger (520-fold) excess of CNBr. At the same time Corradin and Harbury (1970) reported an extensive investigation of the cleavage reaction in 70% aqueous formic acid. The protein was treated with either a 3- or a 50-fold molar excess of CNBr. The fragments 1-80 and 66-104, both still containing a methionine residue, could be isolated after treatment with a 3-fold excess of the reagent. Treatment with 50 equivalents of CNBr also did not give a quantitative

cleavage into three fragments. The fragments 1-80 and 66-104 were isolated, but now in smaller amounts, and, remarkably, contained only internal homoserine instead of methionine.

A mechanism proposed by Carpenter and Shiigi (1974) to account for a similar Met-Hse converion during CNBr cleavage of acetylmethionyl peptides, might be applicable here. They detected O-acetyl homoseryl peptides in the cleavage mixtures by chromatography in acidic solvents. This suggested a mechanism in which a six-membered δ-iminolactone is formed by nucleophilic attack of the carbonyl group of the acetyl moiety on the cyanosulphonium intermediate (Figure 5).

Figure 5



An O-N acyl shift - a smooth reaction in neutral media - would then give the N-acetylhomoseryl peptide.

The conversion may constitute a general side reaction during CNBr cleavage. The extent to which it competes with γ-iminolactone formation, that results in chain cleavage, seems not only to be sequence-dependent, but also to vary with the choice of the acidic reaction medium. This can be concluded from the following experimental data: Wallace and Offord (1979) observed recently that treatment of cytochrome c, protected at the ε-amino functions with the acetimidyl group, with a 120-fold excess of CNBr in 0.1N HCl is far from quantitative, a result which agrees with that of Black and Leaf (1965), who used the same medium. Substantial amounts of the intact chain and of the fragments 1-80 and 66-104 remained, none of them containing any methionine.

However, in agreement with the earlier results of Corradin and Harbury (1970), it was found that a similar treatment in 70% aqueous formic acid as the solvent leads to more extensive cleavage: only an appreciable amount of the fragment 66-104, devoid of methionine, remained under those conditions.

The procedure in which cytochrome c is reacted with only 3 equivalents of CNBr was adopted since both the fragment 1-65 and 66-104 can then be isolated. The reaction is carried out in 70% aqueous formic acid for 24 hours in the dark and at room temperature. The mixture was then diluted with water (10 volumes) and lyophilized.

The separation of fragments of cytochrome c by gel filtration is usually done in acidic media. Aqueous acetic acid (Black and Leaf, 1965; Fisher *et al*, 1973), aqueous formic acid (Corradin and Harbury, 1970) or mixtures of acidic solvents (McGowan and Stellwagen, 1970) have been used. Gel filtration in 0.1M NH_3 as the solvent might have the advantage, however, that the eluate can be monitored spectrophotometrically at wavelengths where the amide group absorbs, so that peptides without aromatic side chains can be detected.

Figure 6 shows a pattern obtained after gel filtration of the mixture of CNBr fragments in 0.1N NH_3. The resolution proves to be poor in this case. The low molecular fraction contains the fragments 81-104 and 66-80. The larger non-heme fragment 66-104 did not elute separately from the heme containing material. Although the crude mixture of fragments is readily soluble in 0.1N NH_3, the purified sequence 66-104 is only slightly soluble in aqueous media above pH 6-7. The poor separation in Figure 8 is, therefore, probably due to aggregation of fragments in 0.1N NH_3.

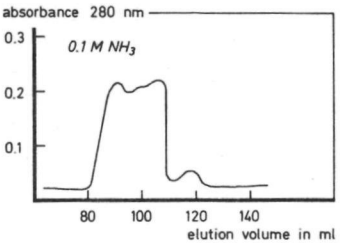


Fig 6 Gel filtration of CNBr cleavage products of cytochrome c (24 mg) on Sephadex G-50 (1.5 x 120 cm); eluent 0.1N NH_3; flow rate 8.0 ml/h.

Figures 7 and 8 demonstrate that aqueous formic acid is a superior solvent for the separation of cytochrome c fragments by gel filtration.

Figure 7A shows the separation in 0.1N NH_3 of the products obtained from a tryptic digestion of maleylated peptide 1-65. The fractions from this separation indicated in the figure were combined and lyophilized. The mixture of maleylated fragments 1-38 and 39-65 was then deprotected and subjected again to gelfiltration on a column, run in 7% aqueous

formic acid (Figure 7B) under otherwise identical conditions as in Figure 7A. The resolution was much better in this case.

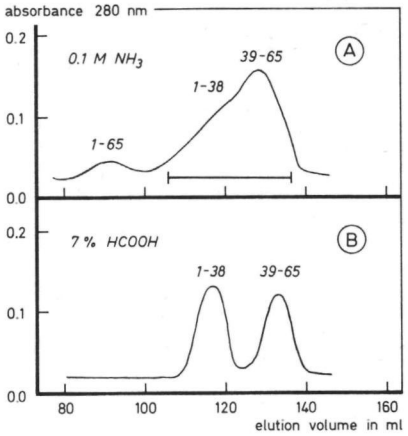
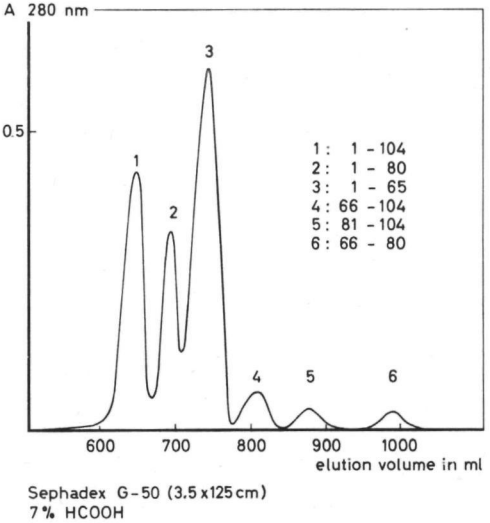


Fig 7 Gel filtration of a tryptic digest of maleylated cytochrome c fragment 1-65 (18 mg) on Sephadex G-50 (1.5 x 120 cm). The separation obtained with 0.1N NH_3 is shown in A; the indicated fractions were pooled and rechromatographed in 7% aqueous formic acid (B); flow rate 7.8 ml/h in both cases.

The separation of the fragments obtained by limited cyanogen bromide cleavage of native cytochrome c, in 7% aqueous formic acid, is illustrated in Figure 8.

Fig 8 Gel filtration of the fragments from a limited CNBr degradation of cytochrome c (130 mg) on Sephadex G-50 (3.5x125 cm); eluent 7% aqueous formic acid; flow rate 29 ml/h.



The elution profile compares favourably with that reported by Corradin and Harbury (1970).

The amino acid analyses of all the crude fragments agreed with the theoretical values. An important observation was that the fragment 66-

104 still contained a residue of methionine at position 80, which is expected to be a prerequisite for correct complexation with the heme fragment 1-65.

The crude heme fragment 1-65 was occasionally rechromatographed on a similar column to remove traces of the fragment 1-80. A final purification was achieved by ion exchange chromatography on CM-cellulose. Figure 9 shows a typical elution profile. This step removes the unreacted form of the peptide, present in amounts of 30-35%, in which the C-terminal homoserine lactone had been hydrolyzed.

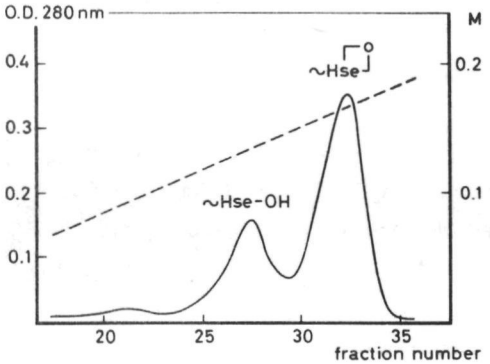


Fig 9 Chromatography of fragment 1-65 (35 mg) on CM-cellulose (1.4 x 28 cm); eluent linear sodium phosphate gradient, 0.01-0.20M, at pH 6.8; flow rate 35 ml/h; fraction volume 10.5 ml.

The two forms of the peptide can be separated on an analytical scale by electrophoresis on cellulose acetate. Moreover, they show different chromatographic mobilities on ascending chromatography on cellogel (Faupel and von Arx, 1976), using a borax-phosphate buffer pH 6.8: the lactone and acid forms of the peptide 1-65 move with Rf=0.68 and Rf= 0.85, respectively, relative cytochrome c (displaced over 5.0 cm after 1.5 h). Analysis of the 1-65 lactone, purified on CM-cellulose, with these techniques revealed the presence of only trace amounts (<5%) of the hydrolyzed form.

A treatment of the crude fragment 1-65 with anhydrous trifluoroacetic acid for 1 hour at 20°, which should convert the homoserine peptide into the lactone form (Ambler, 1965), gave a very non-homogeneous product, as shown by chromatography on CM-cellulose. It exhibited a decreased coupling ability in the reconstitution reaction (cf section 2.3).

The 39-peptide 66-104 was also purified on CM-cellulose (Fig 10). This procedure removed a small amount of a more acidic product, probably formed by deamidation of one of the asparaginyl residues at posi-

tions 70 and 103.

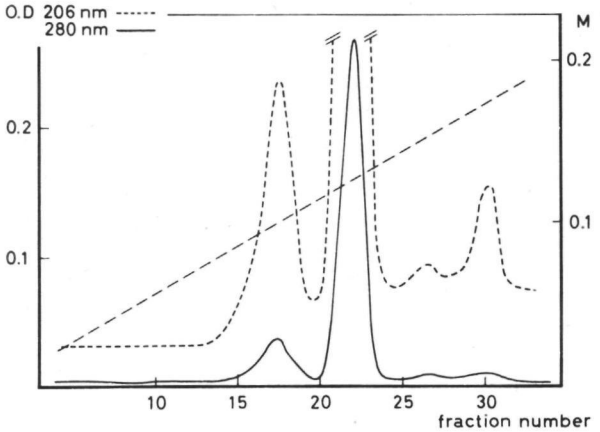


Fig 10 Chromatography of fragment 66-104 (25 mg) on CM-cellulose (1.4 x 28 cm); eluent linear sodium phosphate gradient, 0.01-0.20M, pH 6.8; flow rate 35 ml/h; fraction volume 7.0 ml.

Fractions containing the fragments 1-65 or 66-104 were combined and desalted by gel filtration on Sephadex G-25 in 3% aqueous acetic acid. The pure products were then isolated by lyophilization and stored at -20°.

2.2.2. General properties of the heme peptide 1-65

The visible absorption spectrum of the ferri-heme peptide 1-65 at pH 5.6 exhibits maxima at 406 and 503 nm, but not at 620 nm (Fig 11). These spectral properties are characteristic of a *low*-spin coordination complex, in which two strong-field ligands provided by nitrogen or sulfur containing side chains of the peptide, occupy the fifth and sixth positions of the heme iron. In general, a Soret absorption above 400 nm is indicative of two strong-field ligands, while maxima at 396-400 nm and 390-395 nm indicate the presence of one or two weak-field ligands (oxygen containing groups), respectively (Margoliash and Schejter, 1966). A *high*-spin complex is recognized by an absorption maximum at approximately 620 nm. The ferro-form of the peptide exhibits maxima of 416, 520 and 550 nm.

Babul *et al* (1972) obtained chemical evidence that two histidyl residues in the heme peptide 1-65 are the strong-field ligands. Carboxymethylation of the two histidyl residues was appreciably slower than that of the third one. When complete carboxymethylation was realized, it caused a shift of the Soret maximum from 406 to 390 nm.

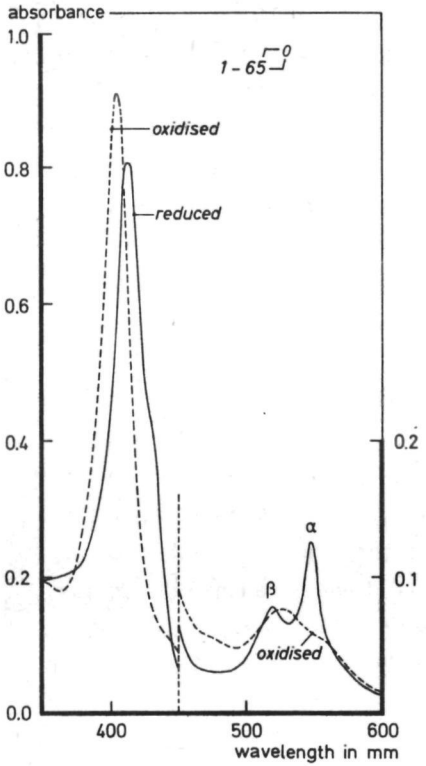


Fig 11 Visible absorption spectra of oxidized and reduced heme peptide 1-65 in 0.1M acetate buffer pH 5.6.

Lowering of the pH of a solution of the ferri-peptide causes a similar shift from 406 to 395 nm. The change from a *low*-spin to a *high*-spin complex, in which one ligand is probably displaced by a solvent molecule, occurs gradually at pH values around 3.8. In contrast the acidic spin-state transition for ferri-cytochrome c shows a very sharp titration curve with an apparent pK_a of 2.5. This low value is attributed to the large number of covalent interactions which stabilize the *low*-spin conformation of the protein. In the heme peptide 1-65 these interactions are largely absent.

Examination of the 550 nm absorption of the ferro-peptide as a function of pH shows a reversible transition centered at pH 4.9 (Ranweiler and Wilson, 1976). This change at 550 nm has been attributed to the formation of a *high*-spin complex by the exchange of one of the axial ligands with a weak-field ligand such as water. Although the decrease in absorption at 550 nm may result from such a ligand exchange, it does not necessarily imply the formation of a *high*-spin complex. Since no accompanying shifts of other absorptions were recorded a change in spin-state seems unlikely. The phenomenon may be compared with the behaviour of ferrocytochrome c below pH 4, where it unfolds but remains in a *low*-spin state, even at pH-values as low as pH 1 (Dickerson and Timkovich, 1975).

Babul *et al* have demonstrated by measurements of ultraviolet solvent perturbation spectra that Trp^{59} in cytochrome c 1-65 is only about one third and the heme moiety about two thirds exposed to the solvent.

Tyr^{48}, buried in the native protein, is readily iodinated under mild conditions in the heme peptide 1-65 and its phenolic hydroxyl group has a normal pK_a of 10.2. In the native protein, however, only one of the four tyrosyl residues present (Tyr^{74} or Tyr^{97}) has pK_a 10.2; the remaining residues have apparent pK_a-values of *ca* 11.5, 12.6 and 12.6 (Stellwagen and van Rooyen, 1967).

The ultraviolet circular dichroism spectrum of the heme fragment 1-65 (Babul *et al*, 1972; Corradin and Harbury, 1971) does not exhibit a minimum at about 220 nm. This indicates the absence of regions having an α-helical conformation. Similar spectra have been measured for thermally unfolded ferricytochrome c (Myer, 1968) and for apocytochrome c (Fisher *et al*, 1973). It is interesting, that the CD-spectrum of porphyrin-cytochrome c (cytochrome c lacking iron) is very similar to that of the native protein. This indicates that the ligation of iron with Hse^{18} and Met^{80} is not required for the folding of the polypeptide chain around the porphyrin system into a compact conformation (Fisher *et al*, 1973); the conformation is thus the result of non-covalent interactions.

The formal potential of the heme-peptide at pH 7.0 was found to be -108 mV *versus* a hydrogen electrode; this value is characteristic for exposed, *low*-spin iron complexes (Wilson, 1974). For the completely exposed heme-peptide 14-21 a formal potential of -207 mV has been measured (Harbury and Loach, 1960). These values deviate strongly from the value + 261 mV, measured for cytochrome c, in which the heme has been buried in a hydrophobic crevice.

The above data indicate that the heme fragment 1-65 has a relatively open, but not a random conformation.

2.3.1. Re-formation of the 65-66 bond

The complexation of the reduced heme-peptide 1-65 with the complementary peptide 66-104 was followed spectrophotometrically by monotoring the increase in absorbance at 550 nm. The condensation was carried out (see Experimental) in a nitrogen or argon atmosphere. In experiments on a preparative scale a mixture of equimolar quantities of the cytochrome c fragments, dissolved in 0.1M sodium acetate buffer, pH 5.6, was kept in the reduced form for approximately 40 hours. The mixture was then applied directly to a Sephadex G-50 column, which was developed with 7% aqueous formic acid.

The homoserine-lactone function represents a weak activation of the

carboxyl group in the 1-65 sequence. At neutral pH aminolysis by arbitrary amino functions can be excluded, and hydrolysis must be a slow process, since repeated ion exchange chromatography of the purified 1-65-lactone leads only to trace amounts (<5%) of the homoserine form. The selective acylation by the amino function of Glu^{66} in the reduced complex can thus be expected to proceed almost quantitatively, since side reactions are virtually absent.

In order to obtain the highest possible yield, in the first experiments the crude fragment 1-65 - obtained after repeated gel filtration on Sephadex G-50 - was treated with pure trifluoroacetic acid for 1 hour at room temperature. This treatment should convert the homoserine form, when still present, into the lactone form (Ambler, 1965). The yields of $|Hse^{65}|$-cytochrome c ranging from 40-50% were, however, significantly lower than in experiments in which crude 1-65, that still contained approximately 30% of the homoserine form, was used (65-70%; Figure 12A). Apparently the trifluoroacetic acid treatment partly reduced the ability for resynthesis, contrary to expectation. The non-homogeneity of the TFA-treated material was demonstrated subsequently by ion exchange chromatography on CM-cellulose, which revealed four additional products.

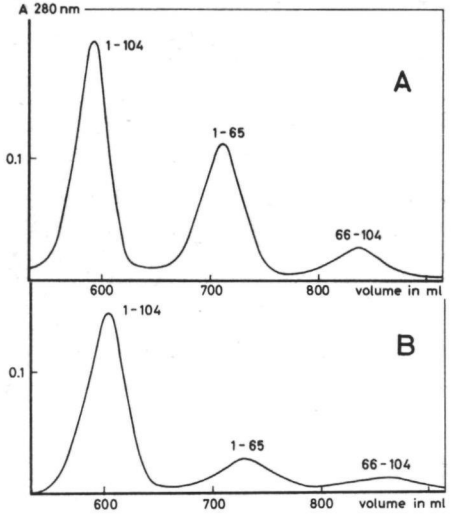


Fig 12 Isolation of Hse^{65}-cytochrome c after reaction between crude (A) and purified (B) fragments 1-65 and 66-104. Sephadex G-50 (3.5 x 125 cm); eluent 7% aqueous formic acid; flow rate 33 ml/h.

It was then decided to purify the fragments 1-65 and 66-104 by CM-cellulose chromatography as described in section 2.2.1. The purified fragments gave crude yields of resynthesis of 85-95% (Figure 12B).

The crude Hse^{65}-cytochrome c was isolated by lyophilization from the 7% formic acid eluates. Treatment of a sample of this material with a large excess of cyanogen bromide gave the fragments 1-80 and 81-104 as the sole products.

Up to this stage the procedure of Corradin and Harbury (1974) had been followed, except for the ion exchange purification of the fragments. The product should thus correspond closely to the latter, for which the need for further purification has not been reported; these authors give electron transfer activities in the cytochrome c depleted mitochondrion assay (Jacobs and Sanadi, 1960; Blair, 1967).

Although the activity of the crude Hse^{65}-cytochrome c in this succinate oxidase system was comparable with that of the native protein (Figure 16), the analogue exhibited only a very modest activity in the reaction with isolated beef heart cytochrome c oxidase (see section 2.4). An accurate determination of the state of purity was therefore desired.

2.3.2. Purification of |Hse^{65}|-cytochrome c

The characteristic properties of the native protein are:

1. the UV-visible absorption spectrum of the ferric protein, particularly the weak maximum at 695 nm, which is indicative of the coordination of methionine-80 to the heme iron
2. the inability of the reduced protein to ligand exchange with carbon monoxide
3. the complete reducibility of the ferric protein by ascorbate.

The crude |Hse^{65}|-cytochrome c showed only a very weak 695 nm band. Reduction with ascorbate was significantly slower than with the native protein and proceeded to an extent of only 62-68%, as compared with reduction by sodium dithionite. Approximately 24% of the reduced ($Na_2S_2O_4$) analogue combined with CO.

The properties clearly indicated the need for further purification. This was attempted by ion exchange chromatography on CM-cellulose, using 0.1M sodium phosphate buffer pH 6.8 as the developing solvent (Dixon and Thompson, 1968). Only 15-20% of the applied product was eluted at the position of the native protein, showed the expected reducibility with ascorbate, and was non-autoxidizable. The fractions that were eluted upon increasing the ionic strength of the buffer contained material that was only partly reducible with ascorbate (65-85%) and reacted with CO to varying extents (24-30%). Approximately 50% of the crude product could not be eluted from the CM-cellulose column with 0.5N NH_3, because it was apparently irreversibly bound.

The native protein, lyophilized from 7% aqueous formic acid, showed a very similar behaviour on CM-cellulose. This is illustrated in Figure 13. A gel filtration step on Sephadex G-25 in 0.1M sodium phosphate buffer pH 6.8 before CM-cellulose chromatography did not lead to a different pattern. The protein behaved homogeneously, however, when a solution of cytochrome c in 7% aqueous formic acid, without having been lyophilized, was passed through the Sephadex G-25 column. This indicates that the combined effects of formic acid and lyophilization cause the inhomogeneity. The denatured forms of cytochrome c probably result from aggregation, very similar to the formation of polymeric species induced by treatment of cytochrome c with ethanol and trichloroacetic acid (Margoliash and Lustgarten, 1962).

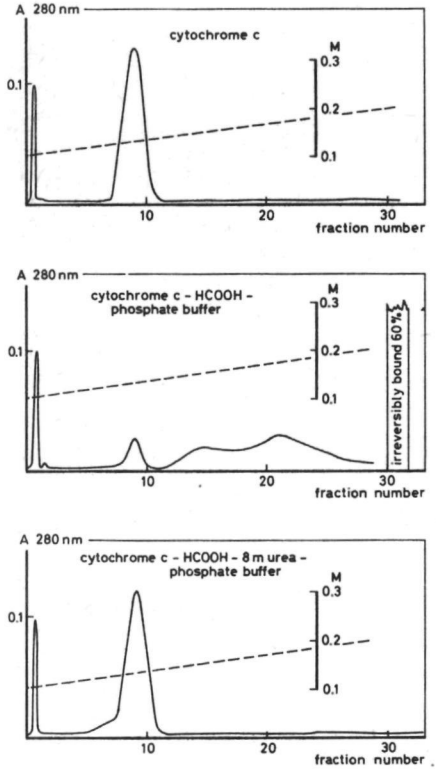


Fig 13 CM-cellulose chromatography using a linear sodium phosphate gradient, 0.1-0.2M, pH 6.8.
Top: native cytochrome c freshly dissolved in 0.1M sodium phosphate buffer, pH 6.8.
Middle: native cytochrome c, lyophilized from 7% aqueous formic acid and dissolved in 0.1M sodium phosphate buffer, pH 6.8.
Bottom: native cytochrome c, lyophilized from 7% aqueous formic acid, treated with urea and dissolved in 0.1M sodium phosphate buffer, pH 6.8.

The formic acid treatment of cytochrome c introduced minor, but characteristic changes in the absorption spectrum of the ferric protein, in addition to the partial loss of the 695 nm band. Blue shifts in the Soret-maximum (from 410 to 408 nm) and in the band at 530 nm (shifted

to 528 nm) were obvious, whilst the fine structure in the ultraviolet region between 260-300 became simplified, mainly due to the loss of a small maximum at 290 nm.

A virtually quantitative renaturation of the formic acid treated cytochrome c was brought about by dissolution in 8M urea, 0.1N in sodium phosphate pH 6.8, followed by gel filtration on a Sephadex G-25 column in 0.1N sodium phosphate buffer pH 6.8 (Figure 13). This procedure, applied to crude |Hse^{65}|-cytochrome c, regenerated the chromatographic properties of the analogue in a similar way as observed for native cytochrome c: a single compound was isolated (Figure 14) at the same elution position as the native protein. The overall yield of |Hse^{65}|-cytochrome c after the treatment ranged from 65-75%.

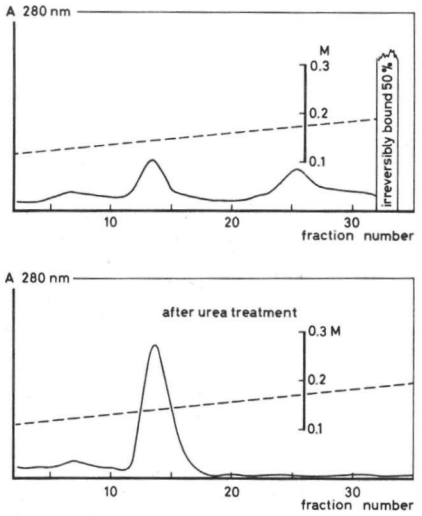


Fig 14 Chromatography of Hse^{65}-cytochrome c, lyophilized from 7% aqueous formic acid, on CM-cellulose before (top) and after (bottom) renaturation with urea. Eluent: linear sodium phosphate gradient, 0.1-0.2M, pH 6.8.

2.2.4. *Physical properties of Hse^{65}-cytochrome c*

The Hse^{65}-cytochrome c, purified as described in the preceding section, was indistinguishable from the native protein in its spectroscopic behaviour, and several chemical properties:

1. the similarity of the ultra-violet and visible absorption spectra includes the presence of a fully developed 695 nm absorption band (Figure 15, curves 1 and 3). Occasionally, however, an additional absorption band, of variable intensity, was observed at about 657 nm, which obscured the presence of the 695 nm band.

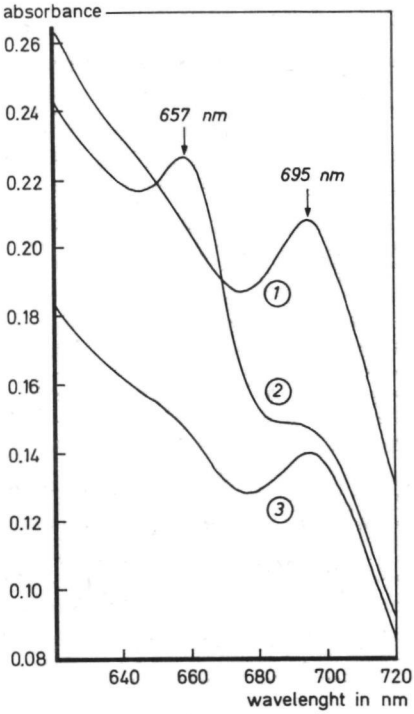


Fig 15 Spectra recorded in the region of 695 nm:
1. native cytochrome c (255 μM, ε530/ε695=13.3)
2. Hse^{65}-cytochrome c (166 μM, ε530/ε695=12.5), prepared in phosphate buffer, pH 7.0
3. Hse^{65}-cytochrome c (171 μM, ε530/ε695=13.4), prepared in acetate buffer, pH 5.6.

The buffer used was 0.2M sodium phosphate, pH 6.8.

The spectrum of a Hse^{65}-cytochrome c preparation with a very pronounced 657 nm absorbance is reproduced in Figure 15. The occurrence of this band was apparently related to the use of 0.1M sodium phosphate buffer, pH 7.0, in the conformationally guided condensation and presumably to the quality of the sodium dithionite that was used. The additional absorbance at 657 nm did not influence any of the other properties of the protein, including the enzymatic activities. When the analogue was prepared in 0.1M sodium acetate buffer, pH 5.6, the phenomenon was only rarely observed. Therefore this latter medium was preferred.

2. The analogue is completely (>98%) reducible with ascorbate. Moreover, this reduction proceeded with the same velocity: pseudo first order rate constants, determined at pH 7.0, 22°, 2.5 mM ascorbate and at a protein concentration of 3.0 μM, were $27 \cdot 10^{-3}$ and $30 \cdot 10^{-3}$ s^{-3} for the native protein and Hse^{65}-cytochrome c, respectively.
3. The reduced form does not bind any carbon monoxide.

These characteristics provide strong evidence that the conformation of Hse^{65}-cytochrome c, with regard to the interactions with the heme moie-

ty, is identical to that of the native protein.

This inference is confirmed completely by the high resolution NMR analysis of the Hse^{65}-cytochrome c (cf Chapter VII, Section 7.5.2.).

2.4. Biological activity of Hse^{65}-cytochrome c

The function of cytochrome c in the oxidative electron-transport chain of eukaryotic mitochondria is that of an electron-carrying shuttle between the two macromolecular complexes III and IV (cf Chapter I, Figure 1). In principle, any part of the chain in which cytochrome c acts as a reductant or oxidant can be used to determine its biological activity.

Unlike other protein compounds of the respiratory chain, cytochrome c can easily be extracted from the mitochondria. The restoration of the oxygen uptake ability of cytochrome c depleted mitochondria, on addition of exogenous cytochrome c, thus constitutes the most obvious way of activity determination. With succinate as the electron donating substrate, this activity is referred to as the succinate oxidase activity. This system has often been used with chemically modified cytochromes c (Margoliash *et al*, 1973). Most of these derivatives, *viz* those modified at residues Trp^{59}, Tyr^{48}, Tyr^{67}, Tyr^{74}, His^{18} and Met^{80}, are defective in a number of the properties of native cytochrome c, including the succinate oxidase activity (Dickerson and Timkovich, 1975; Ferguson-Miller, 1979).

However, this assay system does not seem to be very appropriate for cytochrome c analogues, such as Hse^{65}-cytochrome c, which maintain the structure of the native protein. The main reason is that under the usual conditions of the assay, relatively high ionic strength and cytochrome c concentrations, the electron transfer by cytochrome c does not constitute the rate-limiting step of the entire chain. The slow step in the sequence is one of the reactions in complex III (Smith *et al*, 1974; Nicholls, 1974). This point is illustrated by the observation, that essentially no quantitative differences in reactivity are found when eukaryotic cytochromes c from various species are assayed in cytochrome c depleted rat liver mitochondria (Smith *et al*, 1973), while large differences

are observed in their reactions with cytochrome c oxidase at low ionic strengths and in the presence of various buffer ions (Ferguson-Miller *et al*, 1976). The usefulness of the succinate system was studied in the activity determination of a crude preparation of Hse^{65}-cytochrome c, which was shown to be non-homogeneous with less than 20% of the protein in the native conformation and showed a low reactivity with cytochrome c oxidase (see 2.4.1). Although a rigorous interpretation of the measurements (Figure 16) is difficult because of the low specific activity of

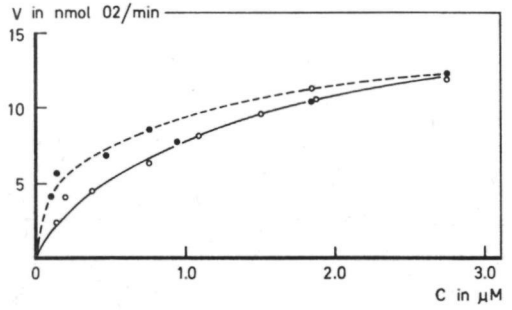


Fig 16 Succinate cytochrome c oxidase activities of native cytochrome c (●) and of a crude preparation of Hse^{65}-cytochrome c (○), that was isolated by lyophilization from 7% aqueous formic acid. Figures have been corrected for the oxygen uptake due to endogenous cytochrome c (mean value: 7.4 nmol O_2/min. Conditions as described in the experimental section.

the mitochondrial preparation and the relatively high basal oxygen uptake, the experiment suggests that the analogue is less active than cytochrome c at concentrations below 1.0 μM, but shows no significant difference in activity at higher concentrations. These observations further demonstrate that the mitochondrial system is not sufficiently sensitive.

Another objection is that any diminished activity does not discriminate between effects on reduction and on oxidation of cytochrome c analogues. It was therefore decided to measure the enzymatic reactions separately: the oxidation by a purified cytochrome c oxidase preparation and the reduction by succinate cytochrome c reductase.

2.4.1. Cytochrome c oxidase activity of Hse^{65}-cytochrome c

The kinetics of the oxidation of cytochrome c by cytochrome c oxidase can be studied in two ways: by spectrophotometric assay of the oxidation of soluble ferro-cytochrome c and by the polarographic measurement of O_2 uptake in the presence of a reductant to ensure that cytochrome c remains fully reduced.

In the spectrophotometric assay the oxidation of ferrocytochrome c

is studied by monitoring the decrease in absorbance of the α (550 nm) β (520 nm) or γ (415 nm) band (Smith and Conrad, 1956).

The time course of the reaction is first order with respect to reduced cytochrome c at all cytochrome c concentrations, as demonstrated by the linearity of plots of $Ln|Ct-C_\infty|$ against time, where Ct and C_∞ are the concentrations of ferrocytochrome c at time t and at completion of the reaction, respectively (Minnaert, 1961; Yonetani and Ray, 1965; Errede *et al*, 1976).

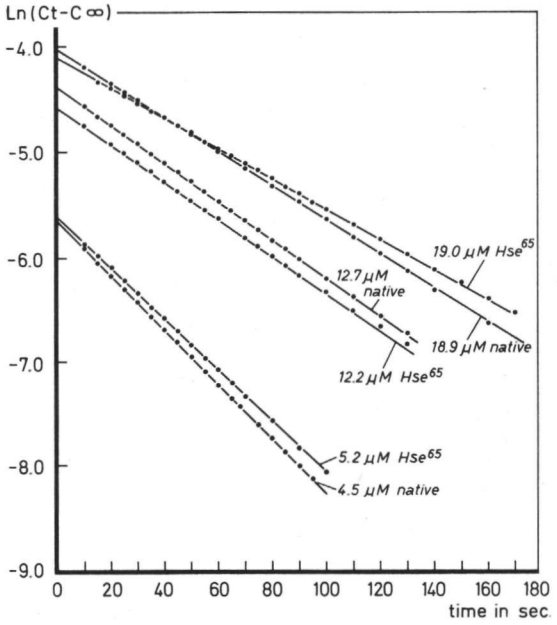


Fig 17 Time course of the oxidase reactions at varying total concentrations of native cytochrome c and Hse^{65}-cytochrome c (see text). Conditions as described in the experimental section.

Figure 17 compares the results of the oxidation of the native protein with that of purified Hse^{65}-cytochrome c at three concentrations. Both proteins exhibited first order kinetics over the entire time range. Moreover, both could be oxidized completely (>95%), as shown by the changes in absorbance upon addition of a small amount of ferricyanide. Two important conclusions can be drawn from Figure 17. First, the linearity of the plots indicate that the Hse^{65}-cytochrome c behaves as a homogeneous preparation. Secondly, the reaction rates are approximately equal for the native protein and the analogue.

The latter observation was quantitated in the following way. The rate equation for the reaction must account for the observation (Smith and Conrad, 1956), that the rate constant (k') is a function of the total concentration of cytochrome c but independent of the initial ratio of ferrocytochrome c and ferricytochrome c. A number of reaction schemes have therefore been proposed (Minnaert, 1961; Yonetani and Ray, 1965)

for which the rate law takes the general form:

$$-\frac{dS}{dt} = v = \frac{A\cdot|S|\cdot|e|}{B+|S|+|P|} \qquad (1)$$

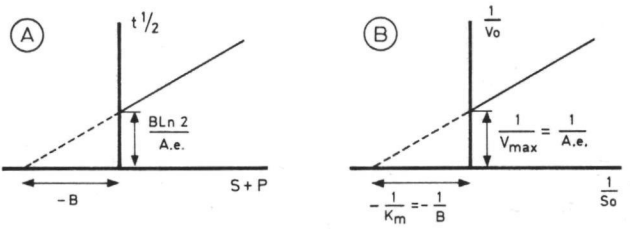
in which $|S|$ = concentration of ferrocytochrome c
$|P|$ = concentration of ferricytochrome c
$|e|$ = concentration of cytochrome c oxidase aa_3
A and B are constants

Integration of (1) gives:

$$\ln\frac{So}{S} = \frac{A\cdot|e|\cdot t}{B+|S|+|P|} \quad \text{and thus:} \quad t_{\frac{1}{2}} = \frac{B+|S|+|P|}{A\cdot e}\cdot\ln 2 \qquad (2)$$

The linear relationship between $t_{\frac{1}{2}}$ and $|P|+|S|$ can be used to evaluate the constants A and B (Figure 18A). A determination of these constants can also be made with the Lineweaver-Burk method, as shown in Figure 18B.

Figure 18



In this case initial velocities have to be used. Since Po=o at t=o, it follows from (1) that:

$$v_o = \frac{A\cdot|e|\cdot So}{B+So} \quad \text{or:} \quad \frac{1}{v_o} = \frac{B+So}{A\cdot e\cdot So} = \left(\frac{B}{So}+1\right)\cdot\frac{1}{A\cdot e} \qquad (3)$$

From the linear plot of $1/v_o$ against $1/So$ it follows that:
B=Km and A=Vmax/|e| = turnover number (TN)

The initial velocities, required to construct the Lineweaver-Burk plot, are calculated from $t_{\frac{1}{2}}$ since direct measurement requires that cytochrome c is fully reduced at t=o.

Combination of Eqns (1) and (2) gives:

$$v = \frac{\ln 2}{t_{\frac{1}{2}}} \cdot |S| \text{ and } v_o = \frac{\ln 2}{t_{\frac{1}{2}}} \cdot |So| \qquad (4)$$

Equation (4) is equivalent to v=kobs·|S|
in which kobs·=k'|oxidase|
(kobs = observed first order rate constant).

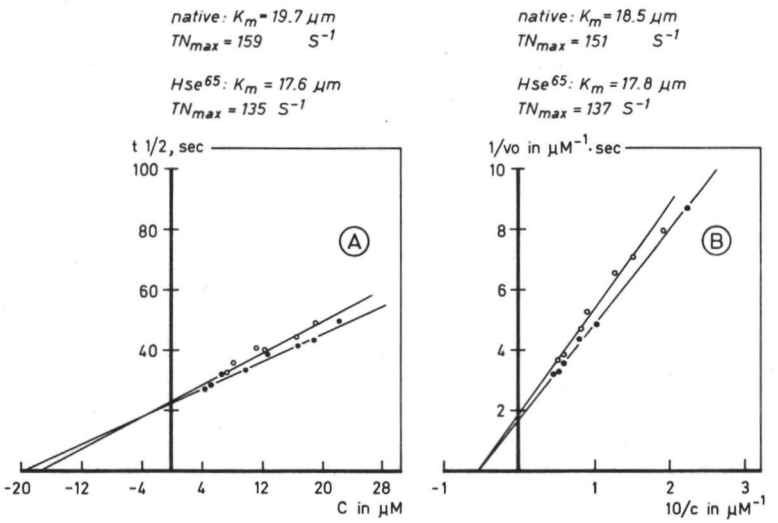


Fig 19 Spectrophotometrically determined activities towards cytochrome c oxidase of native cytochrome c (●) and Hse^{65}-cytochrome c (o). Conditions as described in the experimental section, $t_{\frac{1}{2}}$ values were determined from the time course of the reactions (Figure 17). Kinetic constants were evaluated as indicated in Figure 18.

Figure 19 shows the plots obtained for the native protein and Hse^{65}-cytochrome c. The $t_{\frac{1}{2}}$-values were determined from the plots in Figure 17 representing the time course of the reaction. The kinetic parameters Km and Vmax (Figure 19) appear to be very similar for the two proteins, indicating that they do not differ significantly in reactivity towards the oxidase.

The physical meaning of Km and Vmax depends on the formal mechanism of the oxidation of cytochrome c. A generally accepted scheme is the "Mechanism IV" of Minnaert (1961):

$$c^{2+} + a^{3+} \rightleftharpoons c^{2+} \text{ — } a^{3+} \longrightarrow c^{3+} \text{ — } a^{2+} \xrightarrow[\text{fast}]{O_2} c^{3+} \text{ — } a^{3+} \rightleftharpoons c^{3+} + a^{3+}$$

$$\text{or: } S + E \underset{k2}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k3} EP \underset{k6}{\overset{k5}{\rightleftharpoons}} P + E$$

With k1=k6 and k2=k5 the following rate equation is obtained:

$$v = \frac{k1k2k3 \cdot |S| \cdot |e|}{k1 \cdot (k2+k3) \cdot |P+S| + k2 \cdot (k2+k3)} = \frac{\dfrac{k2k3}{(k2+k3)} \cdot |S| \cdot e}{\dfrac{k2}{k1} + |P+S|} \qquad (5)$$

The assumption that ferricytochrome c and ferrocytochrome c have equal affinities for the binding site on the oxidase is reasonable, since no difference in conformation have been detected for the two forms.

According to equation (5) the maximum turnover number A (=Vmax/e) equals k2k3/(k2+k3) and Km equals k2/k1, *i.e.* the dissociation constant of the cytochrome c-oxidase complex.

Recently Errede *et al* (1976) demonstrated for a purified oxidase preparation that the plot of 1/k' against the total concentration of cytochrome c (equivalent to a $t_{\frac{1}{2}}/|P+S|$ plot) is not linear when measured over a large concentration range (0.7-160 μM). This phenomenon was observed earlier by Nicholls (1965) for the membrane bound enzyme, and explained the spread in the reported values of Km for cytochrome c (1.5-20 μM).

Errede *et al* (1976) gave several modifications of the 'Minnaert IV' mechanism, involving complexes with two molecules of cytochrome c per oxidase. The kinetic constants obtained for selected eukaryotic and prokaryotic cytochrome c agree well with the assumption that the binding to the oxidase, influenced by the cationic residues surrounding the heme edge of the tetrapyrrole rings II and III of cytochrome c, is the dominant parameter in reactivity (Errede and Kamen, 1978).

A comparison of the activities of Hse^{65}-cytochrome c and the native protein, measured polarographically is given in Figure 20. The small differences in the values of Km and TNmax, also observed in the spectro-

photometric assay, probably result from the fact that in all cases single sets of experiments were done.

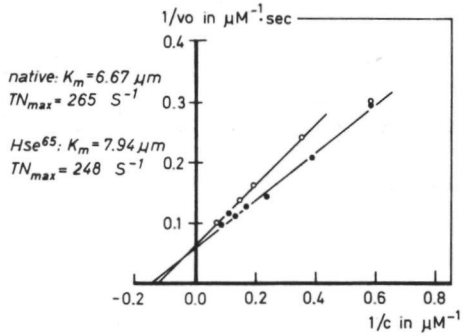


Fig 20 Polarographically determined activities towards cytochrome c oxidase of native cytochrome c (●) and Hse^{65}-cytochrome c (o). Conditions as described in the experimental section.

In Chapter VII (Figure 6A) a comparison of activities, determined with the polarographic method of Ferguson-Miller *et al* (1976) is given. The Km-value determined by that method for Hse^{65}-cytochrome c was again equal to that for the native protein.

It may be concluded that the substitution of Met^{65} by Hse^{65} does not effect the oxidizability of cytochrome c by cytochrome c oxidase.

2.4.2. Succinate cytochrome c reductase activity of Hse^{65}-cytochrome c

The enzymatic reduction of Hse^{65}-cytochrome c was studied with a succinate-cytochrome c reductase (Complex II + III) preparation. The kinetics of this reaction are less well understood than that of the oxidase reaction. The time course of the reduction is composed of a part proceeding at constant rate (zero order part), where the rate-determining step in the reaction-chain is on the substrate site of cytochrome c, and a part where the rate is first order in ferricytochrome c (Smith *et al*, 1974).

The reducibility of Hse^{65}-cytochrome c and the native protein have been compared by measuring initial velocities. The zero order rate of reduction of the native protein ranged from 8.0 to 15.2 nmol s^{-1} with increasing concentrations (5.4 μM to 21.6 μM). The observed zero order rates for Hse^{65}-cytochrome c, at the same concentrations, were between 100 and 118% of those of the native protein.

Hence the substitution of Met^{65} by Hse^{65} in cytochrome c does not noticeably affect the enzymatic reducibility of the protein.

2.5. *Experimental*

Materials: Horse heart cytochrome c was either isolated according to the method of Margoliash and Walasek (1967) or obtained from Sigma (Type III and VI). Commercial samples used for enzymatic activity determinations were purified by ion-exchange chromatography on CM-cellulose (cf Brautigan *et al*, 1978). This procedure was particularly necessary with the Sigma type III preparation, which sometimes contained up to 30% of deamidated material. Sephadex gels were from Pharmacia; CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were from Whatman and equilibrated according to the instructions of the manufacturer.

Cyanogen bromide (Pierce), sodium dithionite (Merck), ascorbic acid (BDH) and N,N,N^1,N^1-tetramethyl-p-phenylenediamine. dihydrochloride (BDH) were used as supplied.

General procedures: Column eluates were monotired with an LKB type III instrument at 280 nm and 206 nm, were appropriate. Amino acid compositions were determined with a modified Jeol JLC-6AH analyser after hydrolysis in 5.7M hydrochloric acid ('Merck', suprapur) for 24 hours at 110-115° in sealed, evacuated ampoules. Spectroscopic measurements were made with a Cary 118 instrument at ambient temperature. Concentrations of cytochrome c were calculated from ΔA_{550} (reduced-oxidized) by using a ΔA of 21.0 mM^{-1} cm^{-1}(van Gelder and Slater, 1962).

Isolation and purification of cytochrome c fragments. Cyanogen bromide degradation of cytochrome c: Cytochrome c (20.0 µmoles, 260 mg) in 70% aqueous formic acid (20 ml) was treated with CNBr (7 mg, 66 µmoles) in the dark for 20 hours at room temperature. The mixture was then diluted with water (200 ml) and lyophilized. The resulting mixture of fragments of cytochrome c in 7% aqueous formic acid was fractionated in 2 equal portions on a Sephadex-G50 column (3.5 x 125 cm). The column was developed with the same solvent at a rate of 30 ml/h. A typical elution pattern is shown in Figure 8.

Appropriate fractions were pooled and lyophilized. Representive yields of fragments from this procedure are: 1-104, 65 mg (~5.3 µmoles); 1-80, 37 mg (~4.0 µmoles); 1-65, 60 mg (~7.9 µmoles); 66-104, 23 mg (~4.9 µmoles); 81-104, 18 mg (~6.4 µmoles). The heme fragment 1-65 was usually re-chromatographed on the same column to remove residual traces of fragment 1-80, giving a final yield of 49 mg (~6.5 µmoles). The fig-

ures indicate that the cleavage at Met^{65} and Met^{80} occurs without any preference.

Purification of fragments 1-65 and 66-104: The heme containing fragment 1-65, isolated from the CNBr digest, is a mixture of the Hse^{65}-lactone form and the hydrolyzed product. The crude fragment (50 mg) was dissolved in sodium phosphate buffer (5 ml; 0.01M, pH 6.8) and applied to a CM-52 column (1.4 x 28 cm), equilibrated in the same buffer. The column was developed at a rate of 35 ml/h with a linear gradient obtained by mixing 200 ml of 0.01M sodium phosphate buffer with 200 ml of the same buffer, but 0.2M in sodium phosphate (pH 6.8). A typical elution profile is shown in Figure 9. Fractions comprising the main peak were pooled and desalted on Sephadex G-25 (2 x 40 cm) with 0.1M acetic acid as the eluent. Lyophilization afforded 32 mg (64%) of the Hse^{65}-lactone fragment.

The complementary fragment 66-104 was purified using exactly the same procedure as described for fragment 1-65. Figure 10 represents a typical elution pattern, obtained when 25 mg of the crude peptide was chromatographed on CM-52 (1.4 x 28 cm). After desalting and lyophilization 19 mg (76%) of the pure fragment was obtained.

Maleylated fragments of cytochrome c: Cytochrome c (45 mg, 3.6 μmoles) in 0.2M borate buffer pH 9.0 (5 ml) was treated with maleic anhydride (105 mg, 1.05 μmoles, 15 equivalents per lysyl residue) at 0° with stirring. The anhydride was added in small portions during 30 minutes while the pH of the solution was kept between 8.5-9.0 by the addition of 0.5N NaOH. The solution was stirred for a further 15 minutes and then desalted on Sephadex G-25 (2 x 40 cm) in 0.1M NH_3. Lyophilization of the eluate yielded 41 mg of the maleylated protein.

Maleyl-cytochrome c (30 mg) dissolved in 0.1M NH_4HCO_3 buffer pH 8.9 (3 ml) was incubated at 37° with trypsin ('Worthington, TPCK-treated'; 30 μl of a 1% solution in 0.001N HCl). After 1 hour a further 30 μl of the trypsin solution was added, and the digestion was continued for a further 2.5 hours. The products were isolated by lyophilization and subsequently separated on Sephadex G-50 (1.5 x 120 cm) in 0.1N NH_3. Figure 6 shows the elution profile obtained from 10 mg of the tryptic digest. Maleylation of the heme fragment 1-65 and its tryptic digestion were carried out in the same manner (cf. Butler *et al*, 1969). Maleyl groups were removed by treat-

ment with 30% aqueous acetic acid for 24 hours at 40°.

Reconstitution of the cytochrome c chain: Hse^{65}-cytochrome c. The 1-65-lactone and the 66-104 fragments were coupled by a procedure similar to that described by Corradin and Harbury (1971) in the assembly depicted in Figure 21. Equimolar amounts of the fragments were dissolv-

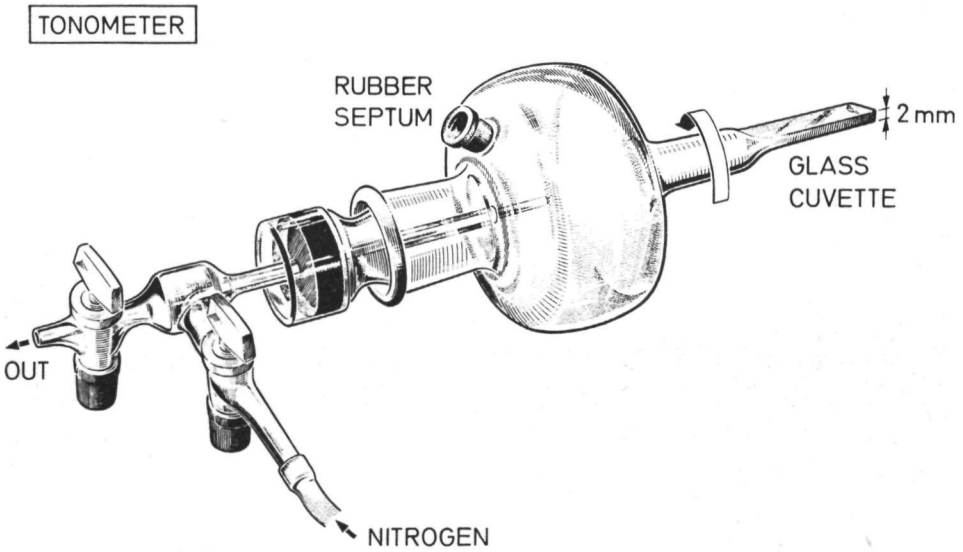


Fig 21 Reaction vessel to carry out reactions under anaeroboc conditions. Deaeration is done by flushing with nitrogen while rotating the assembly horizontally (as shown). Additions are made through the rubber septum by microsyringe injection. The attached glass cuvette allows for spectrophotometric monitoring of the reaction.

ed in 0.1M sodium acetate buffer pH 5.6 - the fragment 66-104 was usually dissolved in the acidic component of the buffer - to give a final concentration of 0.3-0.5 mM of each constituent. The solution was transferred to the reaction vessel, which was then deoxygenated by flushing with highly purified nitrogen. The heme containing peptide 1-65 was reduced by addition of the minimal amount of sodium dithioninte, injected in portions through the septum as a 20 mM solution in deaerated sodium acetate buffer pH 5.6.

The progress of the reduction was followed by measuring the absorbance spectrum (500-600 nm) after each addition of the reducing agent. The resulting solution of the complex 1-65...66-104 was kept in the re-

duced form for 48 hours, the extent of reduction being checked occasionally. The assembly was opened. The decrease in absorbance at 550 nm upon aeration allowed an estimation of the conversion yield (over 90% with purified fragments), since the reduced Hse^{65}-cytochrome c formed is not oxidized by oxygen. The reaction mixture was then subjected to gel filtration on a Sephadex G-50 column in 7% aqueous formic acid. Fractions corresponding to the peak, eluted at the position of native cytochrome c (Figure 12B) were pooled and lyophilized. This material was dissolved in 0.1M sodium phosphate buffer, pH 6.8, containing 8M urea, applied to a Sephadex G-25 column (1.0 x 35 cm) and eluted with 0.1M sodium phosphate buffer, pH 6.8.

The coloured fraction was oxidized with potassium ferricyanide and chromatographed on a CM-52 column in 0.1M sodium phosphate buffer, pH 6.8. The Hse^{65}-cytochrome c eluted as a single symmetrical peak. Appropriate fractions were pooled and diluted once with water. The protein was absorbed onto a small CM-cellulose column, eluted as a concentrated solution with 0.2M phosphate buffer, pH 6.8, rapidly frozen at -78°, and stored at -20°.

Succinate oxidase activity: Mitochondria were isolated from fresh rat liver by the method of Johnson and Lardy (1967). The cytochrome c was subsequently extracted by suspending the mitochondria in a hypotonic medium of 0.015M KCl for 10 minutes (Jacobs and Sanadi, 1960). The mitochondria were kept on ice in 250 mM sucrose and immediately prior to use, resuspended, at a concentration of 15 mg/ml, in the assay buffer, which contained 40 mM potassium phosphate (pH 7.4) and 250 mM sucrose.

The succinate oxidase activity was assayed polarographically at 25° with a Clark O_2-electrode, mounted on a Gilson oxygraph. Assay samples (1.5 ml) were 40 mM in respect to potassium phosphate (pH 7.4), 250 mM in respect to sucrose, 10 mM to succinate and contained 1.0 mg of mitochondria. The rates were expressed as nmoles O_2 uptake per minute after correction for O_2 uptake, due to endogenous cytochrome c.

Cytochrome c oxidase activity: Cytochrome c oxidase (ferrocytochrome c: oxygen oxidoreductase, E.C. 1.9.3.1) was isolated from beef heart by the method of van Buuren (1972; cf procedure III of Hartzell *et al*, 1978) or obtained from Dr B.F. van Gelder (Laboratory of Biochemistry,

B.C.P. Jansen Institute, University of Amsterdam).

The cytochrome aa_3 concentration was calculated from ΔA_{605} (red-ox), using a ΔA of 24.0 $mM^{-1} \cdot cm^{-1}$. The isolated cytochrome c oxidase was diluted in an ice-cold mixture containing 2 mg/ml Asolectine, 0.5% Tween-80, 250 mM sucrose and 10 mM phosphate (pH 7.4).

Spectrophotometric assay: Experiments were performed in 100 mM phosphate buffer (pH 7.3) containing 0.5% Tween-80 and 1 mM EDTA at 25°. Prior to use, a solution of cytochrome c (~200 µM) was reduced by addition of an excess of sodium ascorbate. Excess of the reductant was removed by gel filtration on Sephadex G-50 (1 x 30 cm) and equilibrated with the assay buffer. Buffer and cytochrome c (4-25 µM) were pipetted into 1 ml cuvettes and maintained at 25°. The reaction was initiated by addition of cytochrome c oxidase to give a concentration of 3.8 nM. The disappearance of ferrocytochrome c was monitored by the decrease in absorbance at 550 nm or 415 nm.

Under the conditions of the assay the reaction of the oxidase with oxygen, which was not limiting, was very rapid. The reaction studied was therefore restricted to electron transfer from cytochrome c to oxidase.

Polarographic assay: Oxygen uptake was measured polarographically at 25° with a Clark electrode mounted on a Gilson oxygraph. The reaction mixture (total volume: 1.5 ml) contained: 65 nM cytochrome aa_3, 50 mM potassium phosphage (pH 6.5), 0.5% Tween-80, 250 mM sucrose, 16 mM ascorbate and 1.0 mM TMPD. The reaction was initiated by the addition of cytochrome c (1-7 µM). Rates were expressed as µM cytochrome c oxidized per second after correction for the small rate of oxygen uptake in the absence of added cytochrome c. Neither the native protein nor Hse^{65}-cytochrome c exhibits any autoxodizability. Results are presented in Figure 20.

Succinate cytochrome c reductase activity: The succinate cytochrome c reductase (Complex II + III) preparation was a gift from Dr B.F. van Gelder, and had been isolated according to the method of Yu *et al* (1972).

The reduction of ferricytochrome c (5.4-21.6 µM) was monitored spectrophotometrically by following the increase in absorbance at 550 nm at 25° in the presence of 100 mM potassium phosphate (pH 8.0), 0.5% Tween-80, 20 mM succinate, 0.5 mM cyanide, 1 mM EDTA and 2.0 µg of the reductase (cf Smith *et al*, 1974).

CHAPTER III

MSC-PROTECTED CYTOCHROME C

MSC-PROTECTED CYTOCHROME C

3.1. Introduction

In the scheme, envisaged for the semisynthesis of cytochrome c (Chapter I, Figure 6), the coupling between the fragments 1-65 and 66-104 is the critical step of the chosen strategy. In the preceding chapter a satisfactory procedure, based on 'conformational direction', has been given for this step, and a good method for the purification of the resulting product, the Hse^{65}-cytochrome c, has been described. Moreover, the product is apparently indistinguishable from the native protein in all its functional properties, so that the exchange of Met^{65} for Hse^{65} does not interfere with other structural variations as planned in this study.

With this result the second synthetic problem could be taken up, *viz* the semisynthesis of the cytochrome c sequence 66-104 from a synthetic

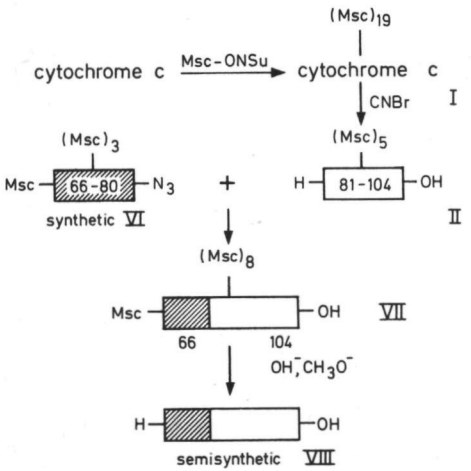


Fig 1 Strategy for the semisynthesis of the cytochrome c sequence 66-104.

fragment 66-80 and the fragment 81-104, isolated from the native protein. It was clear that in this case uniform coupling between the unprotected fragments could not be expected; at least the side-chain amino groups in both fragments had to be protected. For that purpose the Msc-group was chosen as the removable protecting group, whereas the azide

coupling was selected for the condensation of the partially protected fragments (Figure 1).

In this chapter the preparation, isolation and purification of the required N^{ε}-Msc protected sequence 81-104 is described from native cytochrome c.

3.2. The methylsulphonylethyloxycarbonyl (Msc) group

The use of the Msc-group in peptide chemistry was developed some years ago (Tesser, 1975; Tesser and Balvert-Geers, 1975). The group is extremely stable towards acids: it is not attacked by prolonged treatment with pure trifluoroacetic acid or concentrated hydrochloric acid at room temperature and even survives in liquid HF for 30 minutes (Eberle *et al*, 1975). It is hydrolyzed, however, by treatment with 6N HCl for 20 hours at 105-110°C, the conditions used for complete hydrolysis of peptides and proteins. An attractive feature of this urethane-type protecting group is its polar character, imposed by the sulphonyl moiety. As a consequence Msc-protected peptides show higher solubilities in polar solvents, as compared with the more familiar benzyloxycarbonyl- and *t*-butyloxycarbonyl derivatives.

Complete removal of the Msc-groups from peptides is effected by treatment with bases. The reaction proceeds via β-elimination and is very rapid when a hard base is used. With a mixture of dioxan-methanol-4N NaOH (30-9-1), a reagent previously developed to detach peptides from 2-hydroxyethylsulphonylmethyl-substituted polystyrene (Tesser and Ellenbroek, 1967; Tesser *et al*, 1976), removal of the Msc-group is generally completed within seconds (Tesser and Balvert-Geers, 1975). Similarly, treatment with 0.1N NaOH in dioxan-methanol-water (2-1-1) or with 0.05-0.1N $Ba(OH)_2$ (Eberle *et al*, 1975) requires less than one minute for complete removal. The use of $Ba(OH)_2$ enables an easy purification of the products, since the barium ions can be removed as the insoluble sulphate or carbonate.

In fact, the very fast and easy deprotection procedure provided an important argument to prefer the Msc-group to other base-labile protecting groups, *i.e.* the trifluoroacetyl- (Moroder *et al*, 1977) and acetimidyl-group (Wallace and Offord, 1979), for cytochrome c.

3.3. Preparation of Msc-cytochrome c

Methylsulphonylethyloxycarbonyl succinimide (Msc-ONSu) was chosen as the reagent for the introduction of Msc groups on to the side chain amino functions of cytochrome c. This compound offers a considerable advantage over the analogous p-nitrophenyl carbonate, preferred in the preparation of N^{α}-Msc-protected amino acids, in that it is soluble in the mainly aqueous solvent mixtures which are required to dissolve partially or non-protected peptides and proteins. The application of Msc-ONSu also permits the use of gel filtration in aqueous media to separate the high molecular weight products from the excess of the reagent employed, from the N-hydroxysuccinimide liberated during the reaction, and from the organic solvents, mainly DMF, used in the reaction medium.

Initial attempts to derivatize all 19 lysyl ε-amino functions of cytochrome c were performed in 0.5N $NaHCO_3$-DMF (1:1), which has been used previously for the incorporation of Boc-groups (Geiger *et al*, 1971) and Msc-groups (Geiger *et al*, 1975b) into insulin. A large excess of Msc-ONSu, 8 equivalents per lysyl residue, were allowed to react with the protein for 10-15 hours. These conditions correspond approximately to those used generally for modification of protein amino functions. Crude Msc-protected cytochrome c was isolated from the reaction mixture either by gel filtration in 0.1N $NaHCO_3$ or by precipitation of the product on acidification with formic acid or acetic acid.

The analysis of the number of free remaining lysyl residues was achieved by the well-known 1-fluoro-2,4-dinitrophenyl method of Sanger (reviewed by Needleman, 1975): following reaction of the Msc-substituted protein with the reagent, which alkylates unprotected ε-amino groups with concomitant derivatisation of the tyrosyl and histidyl side chain functions, the protein was hydrolyzed completely in acid and the amount of lysine was determined by amino acid analysis. This amount corresponds to the number of lysyl residues that were protected before dinitrophenylation. When the native protein was treated with fluorodinitrobenzene 0.6±0.3 (mean of 6 determinations) residues of lysine per mole of cytochrome c were detectable, so that 95-98% of the 19 lysyl residues present in cytochrome c appeared to be susceptible to reaction with the reagent.

Amino acid analysis after dinitrophenylation of the Msc-cytochrome c

preparation revealed the presence of 17.5-18.5 residues of lysine per mole (mean of 4 determinations). These figures indicate nearly complete protection of the ε-amino functions under the reaction conditions used. Although this result was satisfactory, it was observed that the prolonged exposure of the Msc-cytochrome c to aqueous media at pH 8.0-8.5 during gel filtration in 0.1N NH_4HCO_3, led to some loss of Msc groups. This was also observed by Geiger *et al* (1975a,b) with Msc-insulin.

Any loss of Msc-groups during processing of Msc-cytochrome c results in the liberation of methylvinylsulphone. This alkene alkylates active-hydrogen containing functional groups in the protein, such as ε-amino function of lysyl residues and imidazole groups of histidyl residues, by nucleophilic addition. The alkylation by the analogous ethylvinylsulphone has even been suggested as a method for modifying proteins (Friedman *et al*, 1975). Such irreversible alkylations might also have been occurred during the introduction of the Msc-groups, since this was carried out at pH 8.5 for an extended period.

In view of this observation it was felt necessary to establish more accurately the stability of the Msc-group in aqueous solution and to determine the optimum conditions for the introduction of Msc-groups in cytochrome c.

The stability of the Msc-group in buffered aqueous media was determined by monitoring the loss of the Msc-groups from Z-Lys(Msc)-Lys(Msc)-OH by thin layer chromatography. The model compound was dissolved in various buffers - 0.2M carbonate/bicarbonate pH 9.0-10.5; 0.5M Tris.HCl pH 5.0-9.0; 0.4M NH_4HCO_3 pH 7.9; 0.2M sodium phosphate pH 6.5-8.0 - and kept for periods up to 75 hours. The extent to which the Msc-groups were cleaved proved to be almost independent of the buffer species used, but was dependent on the pH of the buffer solutions. Complete stability of the Msc-group was found only at pH values below 7.5.

At pH 8.0 5-10% of the Msc-group were lost during 15 hours and this figure increased to ca 30% after 75 hours. At pH 9.0 ca 30 and 90% deprotection was noticed afer 15 and 75 hours, respectively. An important result was that 20-30% of the protective groups were lost when the model peptide was kept in DMF-0.5M $NaHCO_3$ (1:1) for 15 hours, since this medium was used in the preparation of Msc-cytochrome c. The lability of the Msc-group to slightly alkaline aqueous conditions is in contrast to

its stability in dry organic solvents: Msc-protected peptides in DMF containing several equivalents of tertiary bases such as triethylamine or ethyldiisopropylamine do not show any deprotection during 15 hours. The main conclusion to be drawn from these observations is that Msc-protected peptides and proteins can only be handled safely for long periods in aqueous solution, *e.g.* during gel filtration and ion exchange chromatography, at pH values below pH 7.5.

The introduction of the Msc-group into lysyl side chains was then studied further with the dipeptide Z-Lys-Lys-OH. The protection of this model compound with Msc-ONSu was found to be complete within 2 minutes, when 8 equivalents of the reagent were supplied in DMF-0.5M $NaHCO_3$ (1:1). When cytochrome c was treated similarly, using 4 equivalents of Msc-ONSu per lysyl residue, full protection was obtained within 30 minutes whereas 17.2, 17.9 and 17.1 residues of lysine per mole of cytochrome c were found after dinitrophenylation for 0.5, 2 and 16 hours, respectively. Since the reaction was apparently much faster than originally anticipated it was decided to follow the course of the reaction more closely, carrying out the reaction under pH control.

Msc-ONSu, dissolved in DMF, was added in small portions to a solution of cytochrome c (salt free) in DMF-water (2:3), whose initial, apparent pH ranged from 9.0-9.4. Each addition of an aliquot of the reagent caused an almost immediate drop in pH, which was restored to a value between 8.0 and 8.5 by addition of ethyldiisopropylamine. The rapid changes in pH were apparently due to the immediate reaction of Msc-ONSu with the amino functions of the protein, which is accompanied by liberation of the weak acid N-hydroxysuccinimide (pK_a=4.04). This conclusion was based on the observation that the hydrolysis of Msc-ONSu in the reaction medium is very slow and that the changes in pH following addition of Msc-ONSu to the reaction mixture became slow when approximately one equivalent of Msc-ONSu per lysyl residue present had been added.

The very efficient protection with Msc-ONSu was verified further by an experiment in which various samples of cytochrome c (30 mg) in DMF-water (1:1, 4 ml) were treated with an increasing number of equivalents of the reagent. After 5 minutes the reaction mixtures were diluted with 0.1 M sodium phosphate buffer, pH 6.9 (1 ml), desalted on Sephadex G-25 in 0.02 of the same buffer, the products were lyophylized, and 2 mg

dissolved in 0.4 ml $NaHCO_3$ solution (10 mg/ml) were subjected to dinitrophenylation by addition of 0.2 ml 5% FDNB in ethanol. The lysine contents, determined by amino acid analyses, are presented in Figure 2.

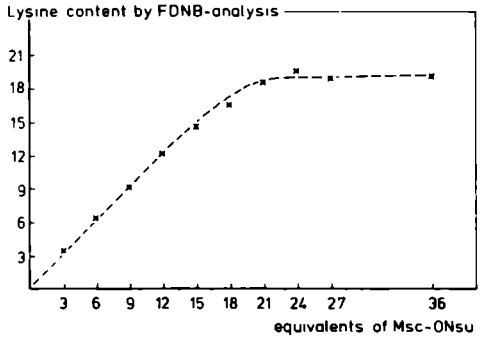


Fig 2 FDNB analysis of the number of ε-amino groups protected on reaction of cytochrome c with increasing amounts of Msc-ONSu. Each point represent a separate experiment (see text).

It appears that the introduction of Msc-groups proceeds almost stoichiometrically with respect to the reagent: approximately 21 equivalents of the reagent suffice to protect all 19 ε-amino functions of the protein. Furthermore it is clear that the reaction proceeds with an unusually high rate, requiring less than one minute for completion.

The effectively complete protection of the amino functions, already indicated by the FDNB analysis of Msc-cytochrome c, was confirmed by tryptic digestion of the derivatized protein. Only two fragments of the protein were obtained, due to cleavage at the Arg^{38}-$Lys(Msc)^{39}$ bond. Apparently the Arg^{91}-Glu^{92} bond is not susceptible to the action of trypsin.

In large scale (1 g) preparations of Msc-cytochrome c by the method described 2 equivalents of Msc-ONSu per lysyl residue were used. The fully N^{ε}-protected derivative was isolated from the reaction mixture by gel filtration on a Sephadex G-25 column in 0.02M phosphate buffer pH 6.9. This step removed all low molecular weight substances as well as the DMF.

The versatility of Msc-ONSu as the reagent of choice to introduce Msc-groups on amino function in *aqueous DMF* was fully confirmed with several peptide derivatives, *i.e.* Z-Lys-Lys-OH, Z-Lys-Lys-Tyr-Ile-Pro-OH (compound *23*, Chapter IV) and ACTH. Full protection is obtained within 2 minutes with a slight excess of Msc-ONSu. The same applies to the preparation of amino acid derivatives, although the suitability is lim-

ited in those cases where the N^{α}-Msc amino acids show a high solubility in water. The reactivity of tyrosine, which fails to react with the p-nitrophenyl carbonate Msc-ONp (Tesser and Balvert-Geers, 1975), towards Msc-ONSu provides a further example (Rajh *et al*, 1980).

3.4. Properties of Msc-cytochrome c

Msc-cytochrome c is soluble only in aqueous media above pH 5.5-6, the exact value depends somewhat on the ionic strength of the medium. The increasing lability of the Msc-group at pH values above 8 implies that the compound should be handled preferably in solution at near neutral pH. Solutions of up to 1-2 mM can then be readily prepared.

Assuming that all carboxylic acid side chains of Msc-cytochrome c, except the propionic acid residues of the heme, are dissociated at pH 7.0 and that both arginines are protonated at this pH, the oxidized protein has a net charge of -10. Consequently, it is completely adsorbed onto DEAE-cellulose from a solution in 0.02 phosphate buffer, pH 6.9. The binding proved to be very tight, since even 0.5M phosphate buffer, pH 6.9, containing 2.0M NaCl did not release the protein from the ion exchange material. Chromatography of Msc-cytochrome c on DEAE-cellulose could be performed, however, in media which were 4M with respect to urea. Msc-cytochrome c in 0.02M phosphate buffer, 4M in urea, was completely adsorbed onto a DEAE-cellulose column. Subsequent development of the column with a sodium phosphate gradient (0.02-0.20M) 4M in urea eluted the protein as a symmetrical band, which contained 95% of the material applied, at approximately 0.09M phosphate. This behaviour suggests strongly that the Msc-cytochrome c was obtained as a single species.

The UV-visible absorption spectrum of the oxidized Msc-cytochrome c, determined after desalting the preparation in 0.02M phosphate buffer, pH 6.9, was very similar to that of the native ferricytochrome c. Especially noteworthy was the presence of a fully developed 695 nm absorption band, which is direct proof of the ligation of the Met^{80} sulfur atom to the heme iron. So, notwithstanding the loss of 19 positive charges at the surface of the molecule, the derivative appears to maintain a conformation which is very similar to that of the native protein. The acidic derivative of cytochrome c shows, however, a decreased ability

to be renatured, since repeated lyophilization led to a gradual decrease in the intensity of the 695 nm band.

A main difference with respect to the native protein was found in the reduction of the analogue with ascorbate, which proceeds approximately 150-200 times slower under identical conditions (0.02M phosphate buffer, pH 6.9; 3.0 mM ascorbate; 15 μM protein). The difference is understandable, since both reactants are negatively charged. The effects of ionic strength on the reduction have not been studied.

Complete reduction of the Msc-cytochrome c was readily achieved with dithionite as the reducing agent. The visible absorption spectrum of the reduced derivative was qualitatively very similar to that of the native protein. The absorption intensities of the Soret, α- and β-bands were comparable with those of the native protein: A_{416red}/A_{410ox}=1.20 (native: 1.22): A_{550red}/A_{280ox}=1.10 (native: 1.25).

3.5. Regeneration of cytochrome c from Msc-cytochrome c

The lability of Msc-protected peptides increases progressively in aqueous solution at pH values above 8, as discussed previously. It appeared that the dipeptide Z-Lys(Msc)-Lys(Msc)-OH was completely deprotected after 15 minutes at pH 10.5 (carbonate/bicarbonate). This treatment provides a mild way of removing Msc-groups, as was also demonstrated by Geiger *et al* (1975a,b) with Msc-insulin: insulin was recovered after 20 minutes in dioxan-water-methanol (1:2:1), pH 10.5. The same authors also carried out a deprotection in dioxan-water (1:1) or DMF-water (1:1) at pH 13, using a much shorter period of 45 seconds, and found that the insulin obtained in this way was very pure. Apparently the 'short-high' method, that was found superior for the removal of Msc-groups from small peptides (Tesser, 1975), is also reliable for the high molecular weight insulin. It was therefore worthwile to attempt this method for the deprotection of Msc-cytochrome c.

Since fully Msc-protected cytochrome c (lyophilized product) is soluble in DMF, we compared the efficiency of the deprotection in DMF-water-methanol (2:1:1) and DMF-methanol (3:1). Methanol was added to the solvent mixtures in order to quench the methylvinylsulphone that was formed, thereby eliminating possible alkylations by this reagent. The β-elimination reaction was initiated by addition of 4N NaOH to give a fi-

nal concentration of 0.15-0.20N (OH^-, CH_3O^-). Although a clear solution was maintained throughout in the water containing system, the addition of base to the initially clear solution of Msc-cytochrome c in the DMF-methanol system caused an immediate precipitation. The precipitate dissolved when the reaction was terminated by addition of acetic acid. Apparently it consisted of the carbamate salt of the product which decomposed in acidic medium.

After removal of the organic solvents and salts by gel filtration of the reaction mixtures on Sephadex G-25 in 0.02M phosphate buffer pH 6.9, the number of amino functions liberated was determined by FDNB analyses. These revealed that deprotection was extremely fast, with both systems: 0.69 and 0.67 residue of lysine per mole of protein were found when the base treatment was stopped after 10-15 seconds and 60 seconds, respectively. These figures indicate that all Msc-groups are essentially split off within 15 seconds.

The UV-visible absorption spectrum of the product isolated from the partially aqueous reaction medium, showed some small deviations from that of the native protein, whilst chromatography on CM-cellulose revealed the presence of some impurities, amounting to 5-10% of the material applied. The product isolated from the DMF-methanol medium had an UV-visible spectrum identical to that of the native protein in every respect and proved to be fully homogeneous upon CM-cellulose chromatography. From these observations it was concluded that a deprotection medium containing a minimum amount of water was preferable for the removal of Msc-groups from large peptides and proteins. During the course of these investigations it was found that DMF could be replaced by DMSO and HMPT.

The cytochrome c, regenerated from the fully protected protein, was reducible by ascorbate at the same rate as the native protein. Moreover, kinetic analysis of the reaction, which went to completion, showed that pseudo-first order kinetics were followed throughout the time course (2.5 mM ascorbate; 15 μM regenerated cytochrome c in 0.02M phosphate buffer pH 6.9). This observation provided additional evidence for homogeneity of the deprotected Msc-cytochrome c.

In conclusion, it appears that the methods used for the incorporation and removal of Msc-groups are not detrimental to cytochrome c in any respect.

3.6. $N^{\varepsilon 86,87,88,99,100}$-*penta Msc-cytochrome c-(81-104)-tetracontapeptide (II)*

The Msc-cytochrome c described in the previous section has been used for the preparation of the selectively N^{ε}-protected sequence 81-104. To that aim Msc-cytochrome c was treated with 70% formic acid with 30 equivalents of CNBr for 24 hours. The mixture of fragments, isolated by lyophilization, could be separated chromatographically on DEAE-cellulose in 4M urea (Figure 3), but the satisfactory separation of the fragments 81-104 and 1-65 became inferior with larger column loads.

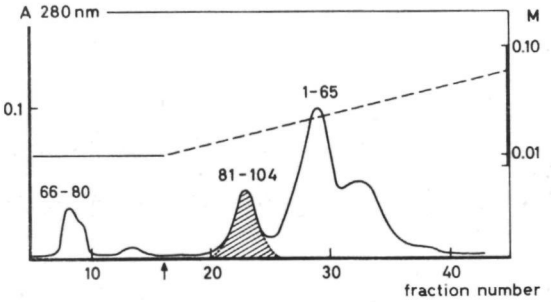


Fig 3 Chromatography of cyanogen bromide fragments from Msc-cytochrome c (20 mg) on DEAE-cellulose (0.8 x 26 cm) in 0.01M sodium phosphate buffer pH 7.0, 4M in urea. Elution with a linear gradient of phosphate (0.01-0.10M, 4M in urea); flow rate: 20 ml/h; fraction volume: 5.0 ml.

It was decided therefore to initially separate the fragments by size. Attempts using Sephadex LH-20, whose exclusion limit should be approximately 5000, with DMF as the solvent were unsuccessful, but gel filtration on Sephadex G-50 in 50% aqueous formic acid as the solvent allowed ready separation of the main fragmentation products 1-65, 81-104 and 66-80. A typical elution profile is reproduced in Figure 4.

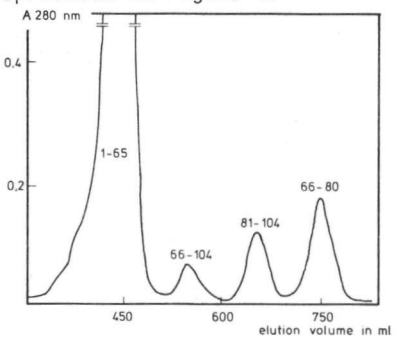


Fig 4 Chromatography of cyanogen bromide fragments from Msc-cytochrome c (250 mg) on Sephadex G-50 (3.5 x 110 cm) in 50% aqueous formic acid; flow rate: 27 ml/h.

It is clear that cleavage at the 65-66 bond was almost quantitative, while the 80-81 bond was cleaved to an extent of approximately 85%.

The fragment 81-104 was subsequently purified on DEAE-cellulose in

Table 1 *COMPARISON OF AMINO ACID ANALYSES[a] OF MSC-CYTOCHROME C (I) AND Nε-MSC-PROTECTED 81-104 (II) WITH THOSE OF THE NATIVE COMPOUNDS*

amino acid		cytochrome c				fragment 81-104				
		native	native FDNB	Msc_{19}	Msc_{19} FDNB	native	native FDNB	Msc_5	Msc_5 FDNB	
Asp	(8)	8.07	8.00	8.17	7.95	2.01	2.07	2.02	2.08	(2)
Thr	(10)	9.14	9.15	9.39	9.48	1.89	1.87	1.88	1.87	(2)
Glu	(12)	11.59	11.65	11.60	11.63	2.91	2.93	2.88	2.91	(3)
Pro	(4)	3.95	3.95	4.49	4.07					
Gly	(12)	11.79	11.87	11.80	11.97	1.07	1.12	1.03	1.05	(1)
Ala[b]	(6)	6.42	6.25	6.43	6.35	3.01	3.01	2.98	2.97	(3)
Cys[c]	(2)									
Val	(3)	3.40	3.23	3.40	3.58					
Met	(2)	1.95	1.78	1.87	2.17					
Ile	(6)	6.04	5.94	5.86	5.95	2.88	2.06	2.99	2.03	(3)
Leu	(6)	6.10	6.29	6.10	6.07	2.01	1.98	2.06	2.04	(2)
Tyr	(4)	3.90	<0.2	3.95	0.48	0.99	0.16	1.00	<0.2	(1)
Phe	(4)	3.96	3.87	3.98	3.98	0.99	0.93	1.01	0.96	(1)
Lys	(19)	18.17	0.34	18.43	18.81	4.73	0.55	4.95	4.93	(5)
His	(3)	3.30	2.52	3.27	1.98					
Arg	(2)	2.14	2.06	2.09	2.00	1.02	1.04	1.03	1.07	(1)

a. No corrections were made for losses during hydrolysis

b. Values found were generally high due to overlap of Ala and Cys

c. Largely destroyed during hydrolysis

4M urea. The elution profile, obtained on developing the column with a linear sodium phosphate gradient in 4M urea (Figure 5), showed that the fragment 81-104 eluted as a single band. The main contaminant, a

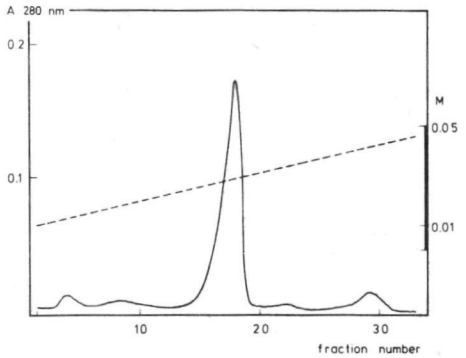


Fig 5 Chromatography of II (115 mg) on DEAE-cellulose (1 x 26 cm). Elution with a linear gradient of phosphate buffer (0.01-0.05M), pH 6.9, 4M in urea; flow rate: 50 ml/h; fraction volume: 12.5 ml.

more acidic derivative of the sequence 81-104, probably contains Asn^{103} as a deamidated residue. This was concluded from the low content of ammonia found during amino acid analyses. The amount of this by-product was dependent on the origin of the cytochrome c used in the preparation of Msc-cytochrome c; one sample of Sigma type III cytochrome c gave over 30% deamidated material, but usually the content did not exceed 10%. The yield of purified, apparently homogeneous II, following desalting on Sephadex G-25 in water and lyophilization, was 45-52%, relative to the starting material, cytochrome c.

Amino acid analyses of the pure fragment, before and after reaction with FDNB (Table I) indicated that all ε-amino functions had been protected by Msc-groups, while the α-amino function of Ile^{81} was available for reaction with FDNB. Finally it was shown that deprotection of II gave a product which, on chromatography on CM-cellulose, behaved similarly to the fragment 81-104, isolated directly from the native protein.

3.7. *Experimental*

As a result of the model experiments described in this chapter, the following optimized procedures for the isolation and purification of the N^{ε}-Msc-protected cytochrome c sequence 81-104 have evolved:

Msc-cytochrome c (I)

Cytochrome c (553 mg, 44.7 µmol) was dissolved in water (20 ml) and DMF

(15 ml). The apparent pH of the solution was adjusted to a value between 8.8 and 9.4 by addition of N-ethyldiisopropylamine (10% vol/vol solution in DMF). Msc-ONSu (440 mg, 1.66 mmol, 2 equivalents per lysyl residue) in DMF (5 ml) was added in portions, while the apparent pH was maintained between 8.5 and 9.0 by manual injection of the base. The reaction mixture was diluted with 1 volume of sodium phosphate buffer (0.02M, pH 6.9) after 10 minutes and subsequently transferred to a Sephadex G-25 column (3.5 x 50 cm) equilibrated and eluted with the same phosphate buffer. The coloured fraction was lyophilized to give 810 mg of the derivitized protein, still containing buffer salts.

A sample (10 mg) of the fully protected derivative I could be deprotected in a mixture of DMF (0.75 ml) and methanol (0.25 ml) by a 15 second treatment with 4M sodium hydroxide (0.050 ml) under vigorous agitation (Vortex). After neutralization with excess acetic acid (0.10 ml), the cytochrome c was recovered by gel filtration on Sephadex G-25 in 0.02M sodium phosphate buffer, pH 6.9.

$N^{\varepsilon 86-88,99,100}$-penta-Msc-cytochrome c-[81-104]-tetracosapeptide (II)

The crude protected cytochrome c, I (800 mg, 44 µmol) was dissolved in 70% (vol/vol) aqueous formic acid (20 ml) and treated with CNBr (140 mg, 1.32 mmol, 30 molar equivalents) for 24 hours in the dark at room temperature. The resulting solution was diluted with water (8 ml) and applied to a Sephadex G-50 column (3.5 x 110 cm) and eluted with 50% (vol/vol) aqueous formic acid. Fractions containing the desired tetracosapeptide (Figure 4) were pooled, concentrated ($<30^{o}$) under reduced pressure to about 2 ml, diluted with 50 ml of water, and lyophilized to give 115 mg of crude II. This material, still containing some formic acid, was dissolved in 4M urea (10 ml) upon addition of a concentrated solution of disodiumhydrogen phosphate, 4M in urea, to pH 7.0. The clear solution was then diluted with 4M urea to give a phosphate concentration of 0.01M, and was subsequently applied to a DEAE-52 column (1 x 26 cm), previously equilibrated in 0.01M sodium phosphate buffer, pH 6.9, 4M in urea. The column was developed with a linear gradient obtained by mixing 250 ml of the 0.01 M buffer with 250 ml of the same buffer 0.05M in sodium phosphate. Material comprising the main peak (Figure 5) was desalted on a Sephadex G-25 column (2.8 x 40 cm) with water as the eluent and recovered by lyophilization (yield: 83 mg, 52%).

CHAPTER IV

SYNTHESIS OF MSC-PROTECTED PEPTIDE HYDRAZIDES OF THE CYTOCHROME C SEQUENCES 66-79 AND 66-80

CHAPTER IV

SYNTHESIS OF MSC-PROTECTED PEPTIDE HYDRAZIDES OF THE CYTOCHROME C SEQUENCES 66-79 AND 66-80

4.1. Introduction

A number of reports have appeared concerning synthetic studies of cytochrome c. Wolman *et al* (1970a,b) and Wolman and Klausner (1971) prepared several protected peptide fragments occurring in the C-terminal region of horse heart cytochrome c. They have also described a synthesis of the cytochrome c-(70-80)-undecapeptide (Wolman *et al*, 1972).

In 1972 Moroder *et al* announced an ambitious plan to synthesize the entire sequence of baker's yeast iso-1-cytochrome c. Detailed descriptions of the preparation of eight fragments spanning all of the 108 residues of the apoprotein have been published (Moroder *et al*, 1973). Three of these fragments were used to assemble the partially protected |Thr^{107}|-baker's yeast cytochrome c-(67-108)-dotetracontapeptide (Moroder *et al*, 1975a).

More recently the same group (Moroder *et al*, 1975b) suggested an approach to the semisynthesis of the horse heart cytochrome c, similar to the scheme described here. The coupling of a synthetic pentadecapeptide (sequence 66-80) with the isolated sequence 81-104, bearing TFA groups on the side chain amino functions, was proposed. A detailed synthesis of the protected pentadecapeptide has since been described (Moroder *et al*, 1977). The partially protected sequences 66-76, 77-87 and 88-104 were also prepared (Borin *et al*, 1976, 1977). No reports have yet appeared, describing the assemblage of the sequence 66-104 from those fragments.

Another completely protected sequence 66-80 of the horse heart protein was prepared by solid phase peptide synthesis (Kirby and Warme, 1978), and used as an intermediate in a semisynthesis of the protein (Nix and Warme, 1976; Ledden *et al*, 1977). Recently, a synthesis of the cytochrome c-(66-104)-nonatriacontapeptide on a polyamide support has been reported by Atherton *et al* (1980).

4.2. Strategy, coupling methods and protective groups

The basic reaction of peptide synthesis involves the formation of an amide bond between an amino-protected amino acid or peptide (carboxyl component) and a carboxyl-protected amino acid or peptide (amino component). Developments in peptide synthesis received a major impetus by the introduction of the benzyloxycarbonyl (Z) group for amino protection by Bergmann and Zervas in 1932. This was followed by the discovery of the *tert*-butyloxycarbonyl (Boc) group by Carpino in 1957, and its application in peptide chemistry by McKay and Albertson (1957), Anderson and McGregor (1957) and Schwyzer *et al* (1959). The benzyloxycarbonyl function can be removed by catalytic hydrogenolysis, whilst the *tert*-butyloxycarbonyl group is stable under these conditions. The latter can be cleaved under relatively mild acidic conditions which do not cleave the Z-group. The complementary nature of these two groups makes them an ideal pair of protecting functions in peptide syntheses where side chain amino groups are also present. Their combined use became very popular, and remains so today.

The enormous increase in the number of possible coupling methods and protective functions during the last few decades is apparent from the extensive compilation by Wünsch (1974). Only a limited number, however, has found general application. A more selective survey of those methods can be found in a review by Finn and Hofmann (1976) and especially in a recent survey of Katsoyannis and Schwartz (1978). The latter review gives a balanced compilation of the now established methods in the synthesis of peptides in homogeneous solution, with many illustrative procedures. An authorative review of the developments in the technique of solid phase peptide synthesis has been written by Erickson and Merrifield (1976). Comprehensive and critical reviews on the major methods of peptide synthesis are provided by Gross and Meienhofer (1979, 1981) in '*The Peptides*: Analysis, Synthesis, Biology'.

Two general methods of synthesizing peptides in solution can be perceived; the stepwise procedure and synthesis by fragment condensations. In the first method the peptide is built up from the carboxyl terminus by sequential acylation and deprotection steps introducing one amino acyl group at the amino terminus in each cycle. This method is often the preferred one for the synthesis of peptides containing not more

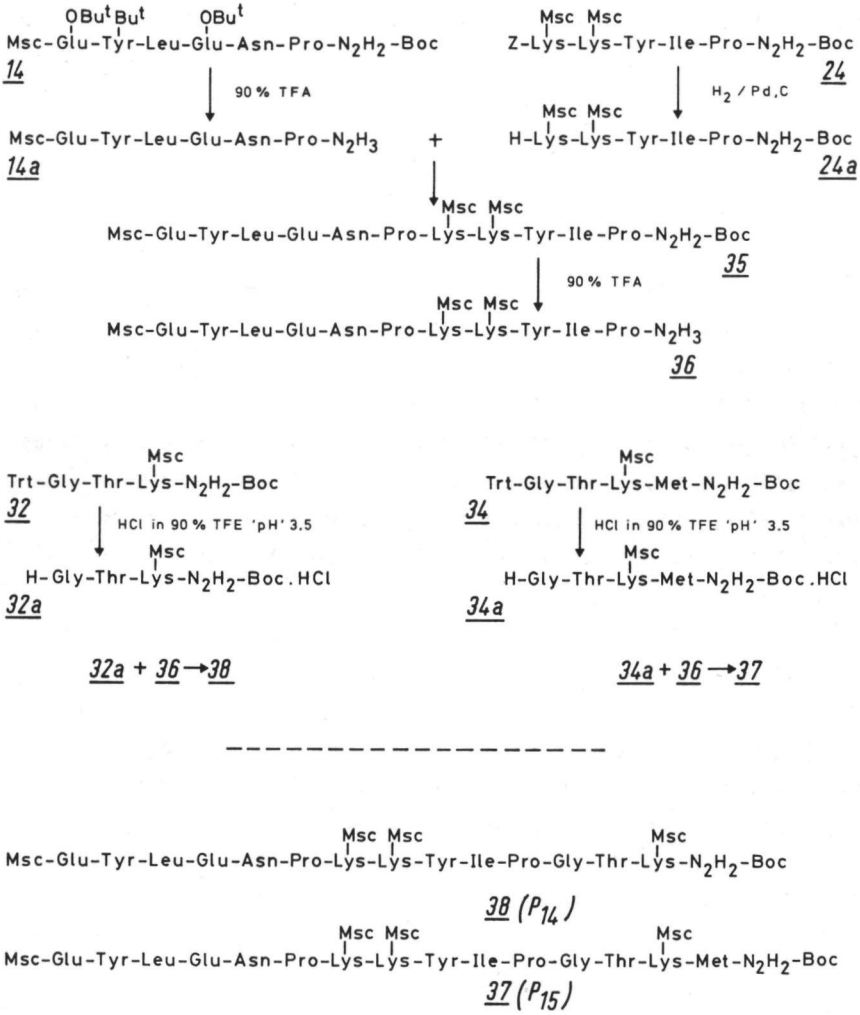
than 10-15 amino acid residues. All the amino acid side chains are usually protected to minimize side reactions.

When the sequence becomes really large (> 30 residues) the differences in physical properties between the starting compound of an acylation step and the product obtained by elongation by one further residue become gradually smaller. This factor together with the diminished solubility of larger, fully protected peptides in organic solvents, severely limits the choice of purification procedures. The synthesis of secretin, containing 27 amino acid residues, illustrates the potential of the method (Bodanszky *et al*, 1967).

It is accepted generally that synthesis by fragment condensation is the method of choice for large polypeptides. The problems of racemisation, negligible in the stepwise procedure when urethane-like groups are used for the protection of activated amino acids, will now limit the number of available coupling methods. Whenever possible, glycine and proline, which cannot racemize, are selected as the C-terminal residues of the fragments. The discovery that the addition of N-hydroxysuccinimide (Wünsch, 1966; Weygand, 1966) or N-hydroxybenzotriazole (König and Geiger, 1970, 1971) to fragment condensations involving peptide bonds other than Gly- and Pro- usually prevented racemization, even so in the polar solvents which are used to dissolve large peptide fragments, added a new and powerful tool to the general strategy. Prior to these developments, the azide method of coupling was believed to be the only one to occur without racemization. More recently, it has been shown, however, that even this method can give rise to racemized products, especially when little care is taken to limit the basicity of the reaction mixture (for a review, see Meienhofer, 1979).

Scheme 1 illustrates the strategy that has been developed for the synthesis of cytochrome c-(66-80)-pentadecapeptide. It is mainly governed by the choice of the Msc-group for the protection of amino functions and by the need to proceed with the end-product in an azide coupling to acylate the natural, Msc-protected sequence 81-104 (see Chapter III). Three protected fragments 14, 24 and 34 were prepared as protected hydrazides. The Z-group in 24 was removed selectively by catalytic hydrogenolysis. Peptide 14 was selectively deprotected by acidolytic cleavage of the *t*-butyl and Boc-functions. The resulting hydrazide 14a was

Scheme 1



activated by conversion into the azide and then used to acylate 24a. The third fragment, 34, was prepared with the acid-labile trityl group as the N^{α}-protecting function, as a Z-group in the presence of methionine can not be removed satisfactorily by catalytic hydrogenolysis. The α-amino terminus of 34 was liberated selectively by a very mild acid hy-

drolysis, that left the Boc-function unaffected. The undecapeptide derivative 35 was converted into the azide *via* 36 and gave the desired pentadecapeptide (P15), 37, on coupling with 34a. The tetradecapeptide (P14), 38, needed in a later stage (Chapter V), was obtained following the same overall scheme, by replacing fragment 34 by the similarly protected tripeptide derivative 32.

4.3. Methods of synthesis

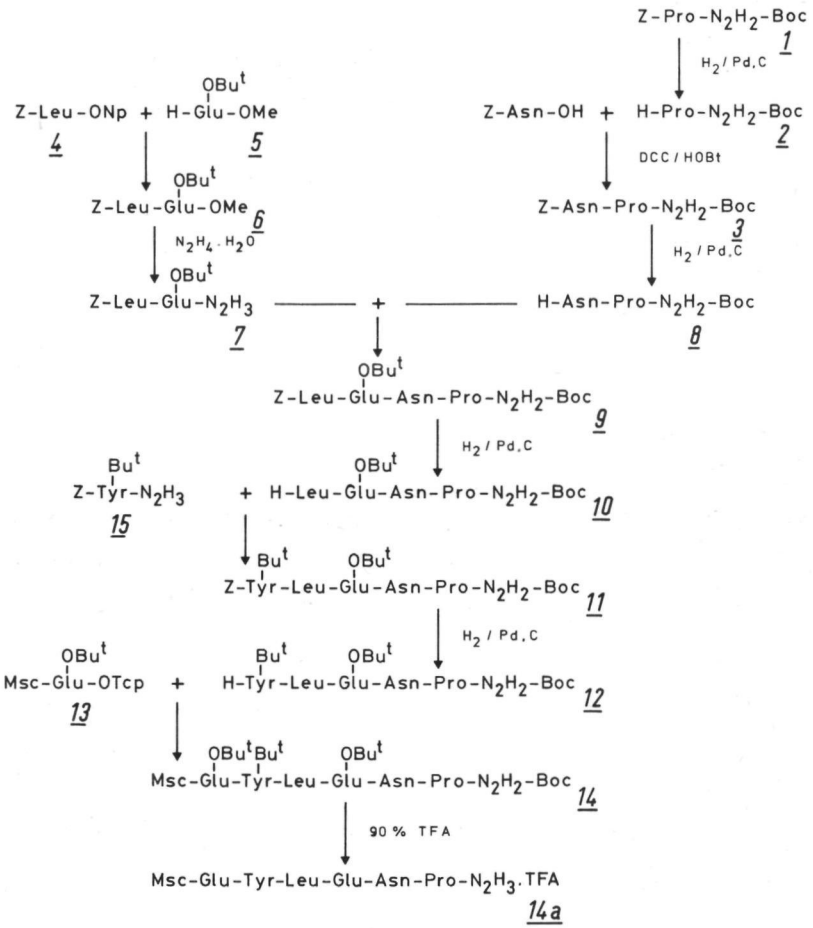
4.3.1. Msc-Glu(OBu^t)-Tyr(Bu^t)-Leu-Glu(OBu^t)-Asn-Pro-N_2H_2-Boc, 14 (Scheme 2)

The synthesis of the fully protected cytochrome c-(66-71)-hexapeptide 14 was initiated with the preparation of 1 from Z-Pro-OH and *t*-butylcarbazate using the mixed anhydride procedure as described by Guttman (1961). Hydrogenolysis of 1 with palladium on charcoal, as the catalyst, gave the deprotected compound 2 which was crystallized from ether. It had physical constants consistent with those given in the literature, but deteriorated when kept for long periods.

The dipeptide derivative 3 was obtained by condensation of Z-Asn-OH with freshly prepared 2 by the method of König and Geiger (1970). An analytically pure compound was obtained following chromatography on silica gel. The infra-red spectrum of the product exhibited no absorption at 2210-2260 cm^{-1} indicating the absence of a β-cyano-alanine group, which would have been formed by dehydratation when carbodiimide alone had been used for the activation of asparagine without side-chain protection.

The glutamic acid derivative 5 was obtained by conversion of Z-Glu(OBu^t)-OH (Buis, 1973; Schröder and Klieger, 1964; Schnabel, 1966) into its methyl ester using diazomethane followed by reductive removal of the Z-function. The acylation of 5 with the *p*-nitrophenyl ester of Z-Leu-OH (4) afforded an 88% yield of the dipeptide methyl ester 6 (cf König and Geiger, 1970), which was treated with hydrazine hydrate in methanol to give the analytically pure peptide hydrazide 7. The condensation of 7 and 8 was achieved by the *in situ* procedure of Honzl and Rudinger (1961). The α-amino function of 9 was liberated by hydrogenolysis to give 10, which was acylated directly with the azide prepared from 15 *via* Rudingers' method.

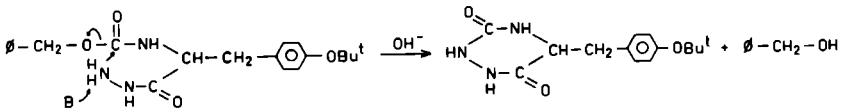
Scheme 2



Purification of the crude, protected pentapeptide 11 by chromatography on silicagel (Hunt and Rigby, 1967) gave an analytically pure, amorphous product in 68% yield.

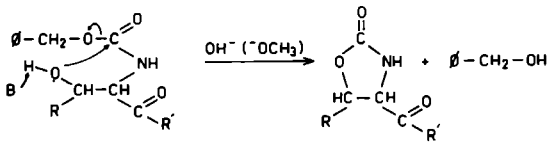
The hitherto unknown hydrazide Z-Tyr-(Bu^t)-N_2H_3 (15) was yielded by hydrazinolysis of the known Z-Tyr-(Bu^t)-OEt. This compound (Schröder, 1963) was prepared from Z-Tyr-OEt (Wünsch and Jentsch, 1964) and isobutene, and invariably contained approximately 10-15% (shown by NMR)

of the starting material. Variation of acid concentration (H_2SO_4) or reaction time did not improve the purity of the product. The pure *t*-butyl ether of the ester could be obtained by silica gel chromatography but it was more convenient to proceed directly to the hydrazide stage with the mixed product. The differing solubilities of the two hydrazides in chloroform afforded a facile purification procedure. Attempts to remove the impurity by extraction of an ethyl acetate solution of the crude hydrazide with 0.2N NaOH were also unsuccessful, since concomitant formation of 3,6-diketo-5-(4'-*tert*butyloxybenzyl)hexahydro-*as*-triazine occurred (see Scheme).



It is presumed that proton abstraction from the hydrazide function induced formation of the *as*-triazine system by subsequent nucleophilic attack on the urethane carbonyl group. This side reaction resembles the basic cyclisation of N-benzyloxycarbonyl-L-serine and -L-threonine derivatives to the corresponding oxazolidine-derivatives (Bergel and Wade, 1959).

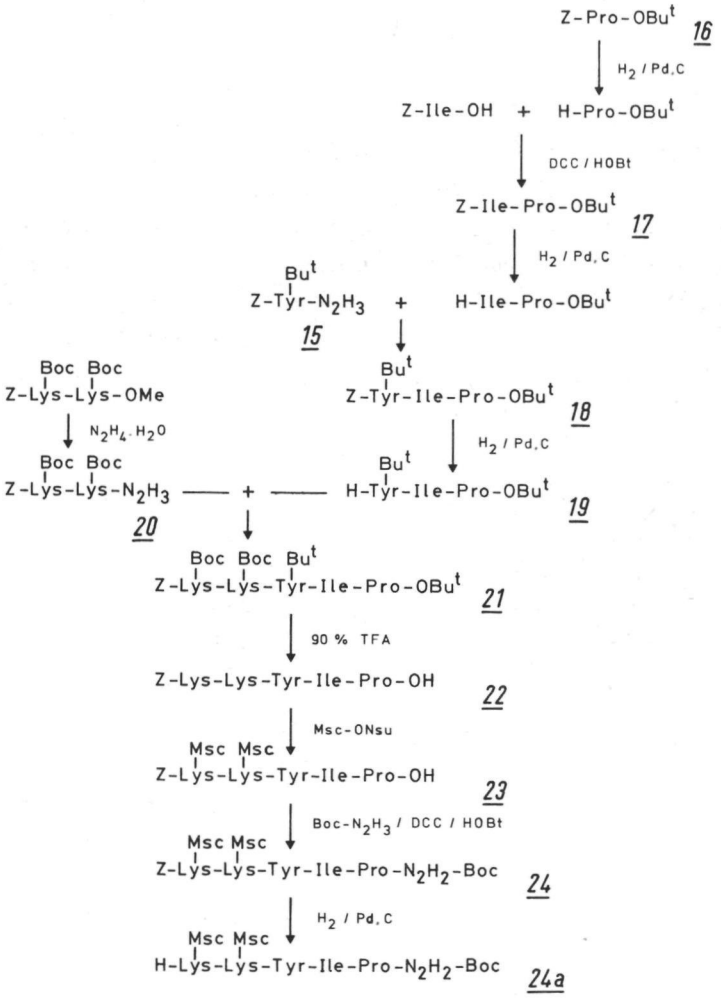
It was shown recently (Stabinsky *et al*, 1978) that this reaction, carried out by treating Z-Ser-OH or Z-Thr-OH in a mixture of 1N NaOH and methanol (1:1) for 2 hours at room temperature, afforded the cyclic products in good yields (70-80%) and, above all, that the reaction occurred without racemisation.



Compound 11 was deprotected at the N-terminus by hydrogenolysis in methanol. The chromatographically pure 12 was subsequently acylated with the 2,4,5-trichlorophenyl ester of Msc-Glu-(OBu^t)-OH (13). The crude hexapeptide 14 was partially purified by precipitation twice from ether. The last traces of amino component 12 could be removed by treatment with Dowex 50W (H^+ form) ion exchange resin. The pure protected

sequence 66-71 (14) was characterized by elemental analysis and NMR-spectroscopy, and by amino acid analysis after complete acid hydrolysis.

4.3.2. Z-Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro-N_2H_2-Boc, 24 (Scheme 3)



Scheme 3

The fully protected sequence 72-76 (21) was constituted by a fragment condensation between the dipeptide azide obtained from 20 and the N^{α}-deprotected tripeptide 18. The synthesis of intermediate 18 commenced with the coupling of Z-Ile-OH with H-Pro-Bu^t aided by DCC (König and Geiger, 1970) and HOBt.

The α-amino group of the pure dipeptide 17 isolated as a colourless syrup, was deprotected by catalytic hydrogenation and then acylated with the azide prepared from 15. Compound 18, isolated in analytically pure form (70% yield) as needles, was subjected to hydrogenolysis to afford the crystalline ester 19, which was acylated with the azide prepared from 20. The fully protected pentapeptide 21 crystallized from ethyl acetate or methanol. The four acid labile, protective groups in 21 were cleaved by 90% aqueous trifluoroacetic acid.

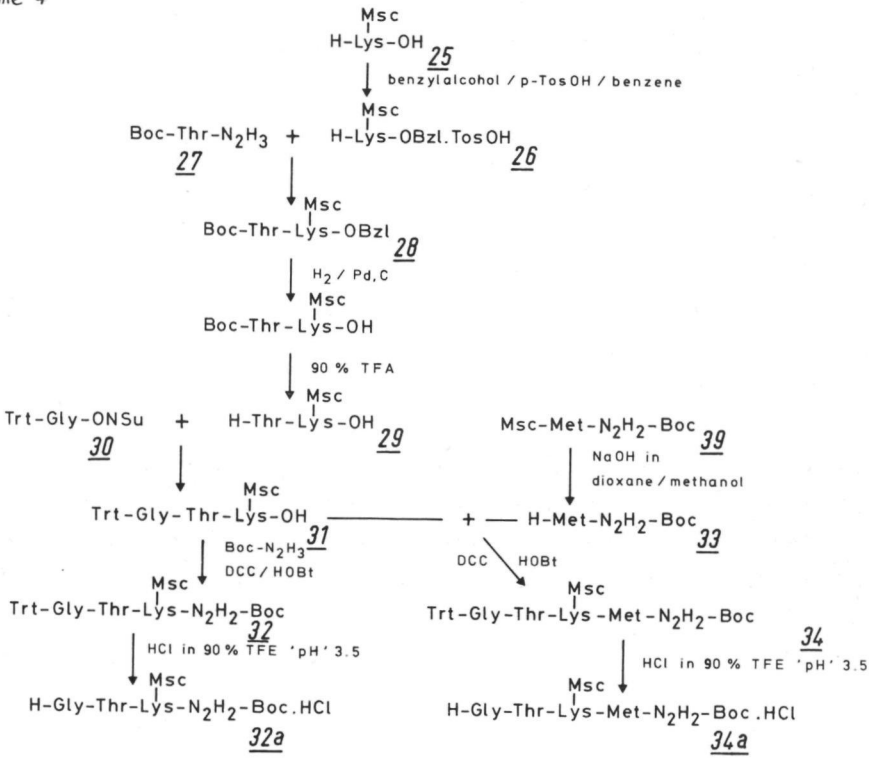
The two ε-amino functions of compound 22 were protected with methylsulphonylethyloxycarbonyl (Msc) groups using the procedure that had been developed for cytochrome c (compare Chapter III). This exchange of protective groups proved to be a straightforward procedure and gave 23 as the monohydrate in an overall yield of approximately 90%. The subsequent conversion of 23 to a protected hydrazide was performed by König's method (1970). The resulting, protected cytochrome c-(72-76)-hexapeptide (24) was obtained analytically pure following silica gel chromatography.

4.3.3. *Trt-Gly-Thr-Lys(Msc)-Met-N_2H_2-Boc, 34 (Scheme 4)*

In the synthetic pathway that was finally adopted for the preparation of this protected 77-80 sequence, the introduction of the C-terminal methionine-80 formed the final step. This strategy had two advantages over the use of H-Met-N_2H_2-Boc as the starting material in a stepwise synthesis. The choice of Z- or Boc-groups for α-amino protection in the acylating moieties was no longer excluded as it would be in the alternative scheme. The risk of sulfoxide formation, always present when working with methionine-containing peptides, was also minimized by the smaller number of manipulations following the introduction of the methionine unit.

The dipeptide 28 was prepared by acylating the benzyl ester of N^{ε}-Msc-Lysine (26) with Boc-Thr-N_3. When the normal conditions for the

Scheme 4



azide formation from the hydrazide 27 were used, *i.e.* treatment in DMF at low temperature with *t*-butyl nitrite for 15 minutes in the presence of 2-3 equivalents of hydrochloric acid (Honzl and Rudinger, 1961), the yields of isolated 28 did not exceed 35%. When a 50% excess of the azide was used the yield increased to about 55%. In each case the crude product was heavily contaminated with a second reaction product, which was isolated by chromatography on silica gel.

The occurrence of side reactions in the azide method has been well documented (for a review see Klausner and Bodanszky, 1974). The Curtius re-arrangement of the azide can lead to the formation of the α-isocyanate derivative of the peptide, which can then react further with the amino component to form a urea linkage. The kinetics of this re-

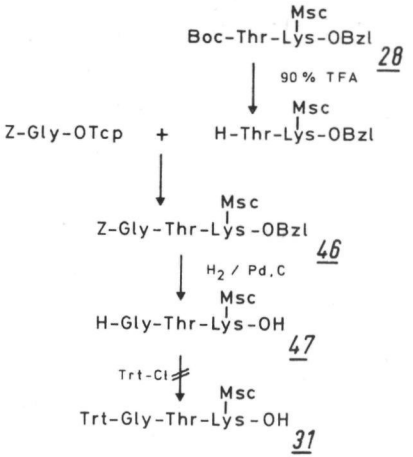
arrangement have been the subject of a recent reinvestigation (Inouye, 1977), which confirmed that this side-reaction can be effectively suppressed by working at low temperatures (0-5°C) and at sufficiently high concentrations of the reactants. These conditions are usually fulfilled.

Amide formation, occurring during the conversion of hydrazide to the azide, has been reported frequently as an additional side-reaction. The assessment of the effects of temperature, solvents, acidity, the type of acid and the nitrosating agent by Honzl and Rudinger (1961), have defined, however, the conditions which minimize this side-reaction. These conditions presently known as the Rudinger method of azide coupling, has been used throughout the work described here.

The additional product that was formed during the synthesis of compound 28 was shown to be the symmetrical disubstituted hydrazide Boc-Thr-NH-NH-Thr-Boc. This assignment was supported by NMR-spectroscopy, elemental analysis figures and by a characteristic chromatographic mobility that differed from that of the authentic amide Boc-Thr-NH_2. The formation of the symmetrical hydrazide is probably to be ascribed to a premature discontinuation of the azide formation, which may be rather slow in this case at the temperature used (-30°C). The presence of unreacted hydrazide in the presumed azide solution will then lead to the side-product. This suggestion was confirmed by extending the reaction time for azide formation to 2 hours. No additional products were then detectable and the desired product 28 was isolated in 77% yield.

In the original scheme for the preparation of 31 (Scheme 5) the synthesis of the zwitterionic tripeptide H-Gly-Thr-Lys(Msc)-OH (47) was envisaged. Deprotection of compound 28 with 90% trifluoroacetic acid and subsequent acylation with Z-Gly-ONSu gave the crystalline, protected precursor 46. Concomitant cleavage of the benzyloxycarbonyl- and benzyl groups by catalytic hydrogenolysis gave compound 47 as a hydrate. Attempts to alkylate 47 with tritylchloride failed, however, to give 31 in an acceptable yield. This was presumably due to the insolubility of 47 in organic solvents and to the rapid hydrolysis of the chloride when water was added as a co-solvent. Transient protection of the functional groups by trimethylsilylation (Kricheldorf, 1970) was also unsuccessful.

Scheme 5



In the alternative approach to the preparation of 31 (indicated in Scheme 4), the benzyl ester function of 28 was removed by hydrogenolysis, followed by acidic cleavage of the N^{α}-Boc-function to give the dipeptide 29. The acylation of 29 in DMF solution with the N-hydroxysuccinimide ester of trityl-glycine (30) gave the desired trityl-tripeptide in high yield (88%).

Removal of the Msc-function from Msc-Met-N_2H_2-Boc, 39, prepared *via* the mixed anhydride obtained from Msc-Met-OH (Tesser and Balvert-Geers, 1975) and isobutylchloroformate, required an extra equivalent of base. This was presumably consumed during proton abstraction from the hydrazide. The resulting intermediate H-Met-N_2H_2-Boc was coupled directly with 31 (König and Geiger, 1970) to yield pure, selectively protected tetrapeptide derivative 34, following chromatography on silica gel.

4.3.4. Trt-Gly-Thr-Lys(Msc)-N_2H_2-Boc (32) and Z-Gly-Thr-Lys(Msc)-N_2H_2-Boc (43)

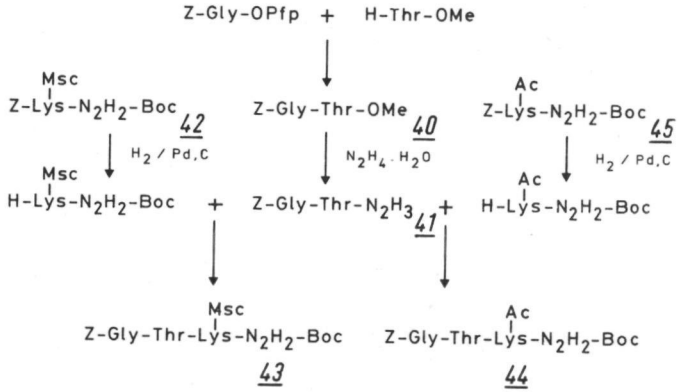
The difficulties that were encountered during attempts to construct the sequence 66-104 by synthesis (Chapter V) from a synthetic sequence 66-80 and the complementary part, obtained from cytochrome c, led to a change in the originally proposed pathway. In the modified scheme the Msc-protected sequence 66-79 was required.

The most convenient way to cope with the deletion of methionine-80,

at the time, was the synthesis of tripeptide 32 (Scheme 4). It was prepared from 31 and *t*-butylcarbazate using DCC and HOBt (König and Geiger, 1970).

In a later stage of the investigation an alternative route to the preparation of the analogous N^{α}-benzyloxycarbonylpeptide (43, Scheme 6) was developed. The choice of 41 as the intermediate gave also access to the N^{ε}-acetyl analogue of the tripeptide (44).

Scheme 6



Moroder *et al* (1973) prepared Z-Gly-Thr-OMe (40) from Z-Gly-ONSu and H-Thr-OMe in moderate yield (49%). The compound 40 was obtained in the present study *via* the pentafluorophenyl ester of Z-Gly-OH and H-Thr-OMe in a rapid and efficient acylation reaction. The product, which crystallized directly from the reaction mixture in 85% yield, was subsequently treated with hydrazine hydrate to give 41.

The desired tripeptides 43 and 44 were synthesized *via* the azide prepared from 41 and the amino components liberated by catalytic hydrogenolysis of compounds 42 and 45, respectively. The lysine derivative 42 was prepared from N^{α}-Z-lysine *via* the Msc intermediate (Tesser and Balvert-Geers, 1975), and subsequent conversion to the protected hydrazide using DCC and HOBt as condensating agents.

4.3.5. Fragment condensations (Scheme 1)

The assembly of the partially protected tetradecapeptide 38 and the analogously protected pentadecapeptide 37 proceeded *via* two successive

azide coupling reactions. The undecapeptide derivative 35 was obtained by acylating the pentapeptide 24a as a free amine with the hexapeptide azide prepared from 14a. Removal of the Boc-group from 35 gave the hydrazide 36, which, after conversion into the corresponding azide, was used to acylate either tetrapeptide 34a or tripeptide 32a. These amino components were obtained by the selective acidolytic cleavage of the trityl-functions from 34 and 32, respectively, using HCl in trifluoroethanol-H_2O (9:1) at a controlled 'pH' (Riniker *et al*, 1975).

All these azide mediated coupling reactions proved to be quite effective. With equimolar amounts of the azide and the amino component the reactions were generally completed within a few hours at 0°, giving conversion yields of more than 85%. The crude products usually contained only a trace of the amino component and an additional, more polar impurity, probably originating from the azide.

The undecapeptide 35 could easily be purified by counter current distribution in a butanol-1-acetic acid-water system. The homogeneous peptide was isolated in 70-75% yield. Purification by exclusion chromatography on Sephadex LH-20 was not effective when DMF-water (9:1) was used as the eluent but elution with 100% DMF separated the impurities from the undecapeptide. Therefore, this technique was preferred in preparations on 100-200 mg scale (actually, LH-20 was only used in the purification of acetylated analogues of the undecapeptide as described in Chapter VI).

The protected pentadecapeptide 37 was isolated from the reaction mixture by chromatography on Sephadex LH-20. Since separation of 37 from 36 was not possible on this gel, the amino component 34a was usually applied in a small excess (10-30%) over the azide. This procedure minimized the amounts of residual undecapeptide in the crude preparations of 37. When necessary, a trace of the methionine-sulfoxide derivative of 37, together with any 36 left, was subsequently removed by counter current distribution in a butanol-1-acetic acid-water system.

Attempts to use Sephadex LH-60, which has a higher exclusion limit than the LH-20 gel, in the purification of 37 failed due to extensive aggregation on this matrix. The peptide 37 was eluted as a very broad band starting at the void volume of the column. This phenomenon has also been observed by Galpin *et al* (1978) and apparently does not occur

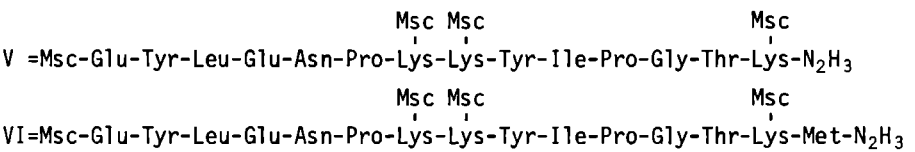
on Sephadex LH-20 or Enzacryl K_2 gels.

Compound 37 behaved homogeneously on TLC and gave the expected amino acid ratios after acid hydrolysis. Complete deprotection of 37 by treatment with trifluoroacetic acid to remove the Boc-group, followed by a short treatment with alkali to split off the four Msc-groups, gave the 66-80 peptide hydrazide, which proved to be homogeneous by thin layer chromatography on silica gel and cellogel.

The completeness of the alkaline deprotection step was established by amino acid analyses of the acid hydrolysates of the peptide. After alkylation of the free amino functions with fluorodinitrobenzene 2.11 and 3.19 residues of glutamic acid and lysine, respectively, per mole of Msc-protected peptide 37 (FDNB treated) were found, while these figures changed to 1.11 and 0.11 for the deprotected peptide. Thus both the α-amino terminus (Glu^{66}) and the three ε-amino groups (Lys^{72}, Lys^{73} and Lys^{79}) were liberated quantitatively by the alkali treatment.

Crude preparations of the tetradecapeptide 38 contained, in addition to the applied excess of the tripeptide 32a, two minor impurities. The amino component 32a was readily removable by acid extraction of a butanol-1 solution of the peptide. A thorough purification proved to be more convenient at the stage where the Boc-protecting group had been split off by trifluoroacetic acid. A chromatographically homogeneous N^{α}, N^{ϵ}-Msc-protected tetradecapeptide hydrazide (V), representing the 66-79 sequence of cytochrome c, was obtained in 60% overall yield following counter current distribution in a butanol-1-acetic acid-water solvent system. The amino acid analysis of V was in good agreement with the expected values.

A treatment of compound 37 with 90% aqueous trifluoroacetic acid afforded the pure N^{α}, N^{ϵ}-Msc-protected pentadecapeptide hydrazide VI, representing the 66-80 sequence.



Peptide derivatives V and VI have been used in semisyntheses of cytochrome c-(66-104)-nonatriacontapeptide (VIII) as described in Chapter V.

Melting points were determined with a Tottolli apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. Elemental analyses were carried out by Mr. J. Diersmann of the micro-analytical department of the Laboratory of Organic Chemistry. Before analysis samples were dried *in vacuo* over P_2O_5 at 40-60°C for 20 hours. Amino acid compositions were determined by Mr. M. v.d. Gaag with a modified Jeol J.L.C.-6 AH analyzer after hydrolysis in 5.7 M hydrochloric acid (Merck, Suprapur) at 110-115°C for 24 hours in sealed, evacuated ampoules. Values for Tyr and Thr have not been corrected for amino acid decomposition. ^{1}H-NMR spectra were recorded by Mr. H. Brinkhof on a Bruker WH-90 spectrometer with TMS (tetramethylsilane, δ = 0.00 ppm) as internal standard.

Ascending thin layer chromatography (T.L.C.) was performed on silicagel 60 F254 plates (Merck, art. 5715). The solvent systems used to develop the plates and the reagents used to locate the amino acid derivatives and peptides are listed in Appendix I. Thin layer chromatography on cellulose acetate was done on Cellogel strips 8 x 17 cm (Chemetron, Milan, Italy) as described by Von Arx and Faupel (1976). Silicagel G-60 (Merck, art. 7731) and silicagel 60 (Merck, 70-230 mesh ASTM, art. 7734) were used for column chromatography. Sephadex LH-20 and LH-60 gels were obtained from Pharmacia.

Hydrogenations were performed at room temperature and atmospheric pressure with palladised (~10% Pd), activated charcoal (Merck-Schuchardt, art. 807104) as the catalyst. Unless stated otherwise, the product from a condensation step was isolated by evaporating the solvents *in vacuo* at a bath temperature of 30-40°C. The residue was then partitioned between ethyl acetate and water. The organic layer was washed successively with 2 M $KHSO_4$, water, 2 M $KHCO_3$ and water and dried (Na_2SO_4).

Z-Pro-N_2H_2-Boc (1)

Z-Pro-OH (37.4 g, 150 mmoles) was condensed with *t*-butyl carbazate (19.8 g, 150 mmoles) using the mixed anhydride method. The procedure described by Guttman (1961) was followed except for the use of isobutyl chloroformate. The product, a colourless syrup, was dissolved in ethyl acetate. It crystallized slowly on addition of diisopropyl ether to turbidity. After storage at 0°C for 6 hours compound 1 was collected by filtration and dried. Yield: 49 g (90%); m.p.: 71-74°C; $[\alpha]_D^{25}$ = -87.0° (c = 1.0, MeOH); TLC : R_f = 0.56 (A), 0.79 (C).
Lit.: Guttman (1961): Yield 89%, amorph from petroleum ether 60-80°; m.p. not well defined; a sample, recrystallized from ether had m.p. 164°; $[\alpha]_D^{21}$ = -83.4 ± 0.5° (c = 1.1, MeOH).

H-Pro-N_2H_2-Boc (2)

Hydrogenation of 1 (10.3 g, 28.5 mmoles) in methanol (200 ml) in the presence of palladium on charcoal (0.5 g) gave, after removal of the catalyst by filtration and concentration of the filtrate, a colourless syrup, which was dissolved in hot diethyl ether. Compound 2 crystallized on cooling. Yield: 4.8 g (74%); m.p.: 117-118°C; TLC : R_f = 0.16 (B), 0.74 (K). The crystalline 2 deteriorated slowly and was therefore prepared freshly when required.
Lit.: Guttman (1961): yield 86%; m.p.: 119°C.

Z-Asn-Pro-N_2H_2-Boc (3)

Dicyclohexylcarbodiimide (6.6 g, 32 mmoles) was added to a mixture of Z-Asn-OH (8.0 g, 32 mmoles), compound 2 (7.32 g, 32 mmoles) and HOBt (4.3 g, 32 mmoles) in DMF (80 ml) cooled to 0°C. The mixture was maintained at this temperature for 16 hours, whereon the precipitated dicyclohexylurea was removed by filtration. Concentration of the

filtrate *in vacuo* gave a syrup which was dissolved in ethyl acetate. This solution was washed successively with 2 M $KHSO_4$, 2 M $KHCO_3$ and water, dried (Na_2SO_4), concentrated to a small volume and added dropwise to a large volume of ether to precipitate crude 3 (12.1 g, 79%). This material was dissolved in ethyl acetate and applied to a column of silica gel (500 g, 10 x 17 cm), which was subsequently eluted stepwise with ethyl acetate containing increasing amounts of methanol (0-30% MeOH). Fractions containing chromatographically pure product were combined and evaporated to give a syrup which was crystallized from ethyl acetate-diisopropyl ether to give compound 3. Yield: 9.9 g (65%); m.p.: 119-123°C; $[\alpha]_D^{23} = -104.0°$ (c = 0.85, MeOH); TLC : R_f = 0.51 (A), 0.60 (B). Analysis: C, 55.18; H, 6.53; N, 14.51%; $C_{22}H_{31}N_5O_7$ (477.51) requires C, 55.34; H, 6.54; N, 14.67%.

Z-Leu-ONp (4)

DCC (10.3 g, 50 mmoles) was added with stirring to a solution of Z-Leu-OH (13.2 g, 50 mmoles) and *p*-nitrophenol (7.0 g, 50 mmoles) in ethyl acetate at 0°C. Stirring was continued for an hour at this temperature and then for a further hour at 20°C. The resulting mixture was filtered to remove dicyclohexylurea, and the filtrate evaporated to yield compound 4 which crystallized from ethanol in 80% yield. M.p.: 89.5-91°C; $[\alpha]_D^{20} = -30.7°$ (c = 2.0, DMF); TLC : R_f = 0.90 (B).
Lit.: Bodanszky and du Vigneaud (1959): m.p. 95°C; $[\alpha]_D^{20}$ = -33.5° (c = 2, DMF).

H-Asn-Pro-N_2H_2-Boc (8)

Catalytic hydrogenation of compound 3 in methanol solution in the usual way gave 8 as a pure colourless oil (100%); $[\alpha]_D^{22} = -90.6°$ (c = 1.0, MeOH); TLC : R_f = 0.29 (L).

OBu^t
|
Z-Leu-Glu-N_2H_3 (7)

Z-Glu(OBu^t)-OH (30 mmoles; Buis, 1973) was esterified in ether with diazomethane. The excess of the reagent was destroyed by addition of a few drops of acetic acid. Evaporation of the mixture gave pure Z-Glu(OBu^t)-OMe as a syrup [TLC: R_f = 0.78 (B)], which was subjected to catalytic hydrogenation in methanol (50 ml) in the presence of palladium on charcoal (0.5 g) and acetic acid (1.65 ml). Filtration of the resulting solution through a layer of Hyflo and evaporation of the filtrate gave chromatographically pure H-Glu(OBu^t)-OMe,HOAc as a syrup [29 mmoles, TLC: R_f = 0.35 (C)] which was dissolved in DMF (150 ml) and treated with Z-Leu-ONp (4, 10.0 g, 26 mmoles) for 16 hours at room temperature. The mixture was evaporated *in vacuo* and the residue dissolved in ethyl acetate. The solution was washed successively with 0.1 N HCl, 0.5 M $NaHCO_3$ and water and dried (Na_2SO_4). Evaporation of the organic layer gave 6 as a homogeneous [TLC: R_f = 0.77 (B)] syrup [22.8 mmoles, 88%| (lit.: König and Geiger, 1970: 79% ; m.p.: 96-98 C; $[\alpha]_D^{23}$ = -54.1° (c = 2.1, MeOH)]. The dipeptide ester 6 in methanol (50 ml) was then treated with hydrazine hydrate (4.86 ml, 100 mmoles) at room temperature for 2 days. The mixture was poured into water to give an oil, which solidified on trituration. The hydrazide was collected by filtration, washed with water, dried, and recrystallized from ether. Yield: 7.51 g (71%); m.p.: 107-109°C; $[\alpha]_D^{20}$ = -26.2° (c = 1.0, MeOH); TLC: R_f = 0.58 (B). Analysis: C, 59.39; H, 7.87; N, 12.09%; $C_{23}H_{36}N_4O_6$ (464.55) requires C, 59.47; H, 7.81; N, 12.06%.

OBu^t
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Z-Leu-Glu-Asn-Pro-N_2H_2-Boc (9)

To a cooled (-15°C) solution of 7 (6.0 g, 13 mmoles) in DMF (130 ml) were added with stirring 4.5 N HCl in ethyl

acetate (7.8 ml, 15.5 mmoles) and *tert*-butyl nitrite (1.75 ml, 15.5 mmoles). After 10 minutes at -15°C the solution was neutralized by addition of triethylamine (5.0 ml, 35.7 mmoles). The resulting mixture was then treated with a solution of compound 8 (13 mmoles) in DMF (60 ml) previously cooled to 0°C. After a further period of 16 hours at 0°C the mixture was filtered and the filtrate evaporated *in vacuo*, dissolved in ethyl acetate and processed as described earlier. Dropwise addition of the ethyl acetate solution, concentrated to a small volume, to petroleum ether, 40-60°, with stirring precipitated pure 9 (9.0 g, 89%). M.p.: 105-126°C; $[\alpha]_D^{22}$ = -88.3° (c = 1.0, MeOH); TLC: R_f = 0.63 (B), 0.70 (C). Analysis: C, 56.72; H, 7.32; N, 12.19%; $C_{37}H_{57}N_7O_{11}.\frac{1}{2}$ H O (784.90) requires C, 56.61; H, 7.45; N, 12.49%.

OBu^t
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H-Leu-Glu-Asn-Pro-N_2H_2-Boc.HCl (10)

Catalytic hydrogenation of compound 9 in methanol gave 10 as the free base. The mixture was treated with one equivalent of dry hydrogen chloride, dissolved in ethyl acetate, to flocculate the colloidally dissolved catalyst. Filtration of the mixture and evaporation of the filtrate gave 10 as a chromatographically pure oil. TLC: R_f = 0.08 (B), 0.53 (C).

Bu^t OBu^t
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Z-Tyr-Leu-Glu-Asn-Pro-N_2H_2-Boc (11)

Following the procedure applied in the synthesis of 9, 3.85 g (10 mmoles) of Z-Tyr(Bu^t)-N_2H_3 (15) were converted into the azide and then coupled with 10 (10 mmoles). The crude product, 7.73 g (78%), was purified by column chromatography on silica gel. The column was developed in a stepwise manner with ethyl acetate, containing increasing amounts of methanol (0-20%). Pure 11 was obtained in an amorphous state by precipitation with petroleum ether, 40-60°, in 68%

yield (6.47 g). It did not have a characteristic m.p. (110-130°C); $[\alpha]_D$ = -75.7° (c = 1.0, MeOH); TLC: R_f = 0.65 (B), 0.84 (C), 0.52 (M). Analysis: C, 59.41; H, 7.74; N, 11.00%; $C_{50}H_{64}N_8O_{13}.1\ H\ O$ (1013.18) requires C, 59.27; H, 7.56; N, 11.06%.

Bu^t OBu^t
H-Tyr-Leu-Glu-Asn-Pro-N_2H_2-Boc (12)

Catalytic hydrogenolysis of the benzyloxycarbonyl group of 11 gave chromatographically homogeneous 12 in quantitative yield. TLC: R_f = 0.68 (C). The amorphous free base was converted directly into the hexapeptide hydrazide 14.

OBu^t
Msc-Glu-OTcp (13)

DCC (3.9 g, 19 mmoles) was added with stirring to a solution of Msc-Glu(OBu^t)-OH (6.3 g, 18 mmoles; Tesser and Balvert-Geers, 1975) and 2,4,5-trichlorophenol (19 mmoles) in DMF (60 ml) cooled to 0°C. After 3 hours at 0°C the precipitated DCU was removed by filtration, and the filtrate was evaporated to give a syrup, which crystallized spontaneously. Recrystallization from propanol-2 gave 13. Yield: 7.8 g (81%); m.p.: 116-118°C; $[\alpha]_D^{20}$ = -24.9° (c = 1.43, MeOH). Analysis: C, 42.85; H, 4.62; N, 2.53; S, 5.81; Cl, 19.86%. $C_{19}H_{24}NO_8SCl_3$ (523.83) requires C, 42.85; H, 4.54; N, 2.63; S, 6.02; Cl, 19.96%.

OBu^t Bu^t OBu^t
Msc-Glu-Tyr-Leu-Glu-Asn-Pro-N_2H_2-Boc (14)

Compound 12 (3.44 g, 4.0 mmoles) in DMF (40 ml) was treated with compound 13 (2.4 g, 4.5 mmoles) at 0°C for 24 hours. The mixture was then concentrated *in vacuo* to a small volume and the crude product obtained by precipitation with excess ether. This procedure was then repeated to remove further amounts of trichlorophenol and unreacted

ester 13. The product, containing small amounts of the amino derivative 12 was dissolved in methanol-water (2:1) and passed through Dowex-50W (H^+ form) ion exchange resin. The resin was washed with the same solvent mixture, and the combined eluate and washings were concentrated to small volume and treated with excess ether to give compound 14 as an amorphous, but chromatographically homogeneous solid (4.0 g, 84%); m.p.: 160-161°C; $[\alpha]_D^{24}$ = -69.2° (c = 1.0, MeOH); TLC: R_f = 0.57 (B). Analysis: C, 54.86; H, 7.62; N, 10.42; S, 2.79%. $C_{55}H_{89}N_9O_{18}S.\frac{1}{2}\ H_2O$ requires C, 54.80; H, 7.53; N, 10.46; S, 2.66%. Amino acid analysis after total acid hydrolysis: Asp, 1.04; Glu, 1.94; Pro, 1.02; Leu, 1.03; Tyr, 0.99.

Mso-Glu-Tyr-Leu-Glu-Asn-Pro-N_2H_3.TFA (14a)

The hexapeptide derivative 14 (0.90 g, 0.75 mmoles) was added with stirring to 90% aqueous TFA (10 ml) at -10°C. After approximately 15 minutes a clear solution was obtained, which was then kept at room temperature for 30 minutes. A fine white precipitate of compound 14a was obtained after dropwise addition with stirring of ice-cold ether (200 ml) to the reaction mixture. The product was collected by centrifugation, washed three times with ether and then dried *in vacuo* over KOH pellets. It was shown to be homogeneous by TLC [R_f = 0.21 (C), 0.42 (I)]. Yield: 0.76 g (96%); m.p.: ~115°C (dec.).

Bu^t
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Z-Tyr-N_2H_3 (15)

The free ester H-Tyr-OEt (39.5 g, 189 mmoles) was converted into the carbobenzoxy derivative by the procedure of Wünsch (1974). Recrystallization (EtOAc-petroleum ether, 40-60°) gave 60 g (93%) of the required product; m.p.: 76-77°C; $[\alpha]_D^{20}$ = -4.0° (c = 2.1, ethanol); TLC: R_f = 0.73 (A),

0.86 (C).
Lit.: Wünsch and Jentsch (1964): m.p.: 88-90°C; $[\alpha]_D^{20}$ = -4.5 ± 0.5° (c = 2, ethanol); Bergmann and Zervas (1932): m.p.: 78°C; $[\alpha]_D^{25}$ = -4.7° (ethanol).

Z-Tyr-OEt (50 g, 145 mmoles) in dichloromethane (120 ml) was treated with isobutene (100 ml) and conc. H_2SO_4 (1.0 ml) for 3 days at room temperature (Schröder, 1963) to yield Z-Tyr(Bu^t)-OEt (57.3 g, 99%) as a thick colourless syrup, shown by NMR to be contaminated with some 10-15% of the starting compound [TLC: R_f = 0.82 (A)]. The impure product (47 g, 117 mmoles) in ethanol (100%, 150 ml) was treated with hydrazine hydrate (22 ml, 4 equivalents) at 0°C for 2 days. The solution was concentrated to give an oil. Repeated crystallizations from ethanol/water did not remove the contaminating Z-Tyr-N_2H_3. When crude 15 was dissolved in chloroform, Z-Tyr-N_2H_3, which proved to be only slightly soluble, was removed by filtration. The chloroform was evaporated and the residue was crystallized from ethanol-water (1:1) or from ethyl acetate-petroleum ether, 40-60° (1:3) to afford 15 as fine needles. Yield: 32.0 g (71.4%); m.p.: 117-118 C; $[\alpha]_D^{22}$ = -2.4° (c = 1.33, MeOH), +6.8° (c = 1.1, $CHCl_3$); TLC: R_f = 0.61 (A), 0.61 (B); Z-Tyr-N_2H_3: R_f = 0.44 (A). Analysis: C, 65.24; H, 7.00; N, 10.88%. $C_{21}H_{27}N_3O_4$ (385.46) requires C, 65.44; H, 7.06; N, 10.90%.

A sample of the crude hydrazide 15, contaminated with Z-Tyr-N_2H_3 (ca. 15%) was dissolved in ethyl acetate and the solution was rapidly extracted twice with 0.2 M NaOH at room temperature and neutralized immediately by washing with 0.5 M $KHSO_4$. TLC revealed that this treatment was effective in extracting the phenolic hydrazide, but that a further impurity had been introduced. The ethyl acetate solution was then washed with water, dried (Na_2SO_4) and concentrated to give a syrup, which was dissolved in warm ethanol-water (1:1). On slow cooling a crystalline mass was obtained, that consisted of two types of crystals, small needles and small beads. The crystals were collected by filtration and

then dried. The beads were isolated by washing with ethyl acetate, which dissolved the needles of 15. The additional product, formed during the extraction procedure with alkali, was obtained in 10% yield. It was characterised by elemental analysis and NMR as 3,6-diketo-5-(4'-tert.butyloxybenzyl)-hexahydro-*as*-triazine. M.p.: 186-188°C; TLC: R_f = 0.54 (A). Analysis: C, 60.83; H, 7.10; N, 15.04%. $C_{14}H_{19}N_3O_3$ (277.32) requires C, 60.63; H, 6.91; N, 15.15%; NMR (DMSO-d^6): 1.27 (s, 9H, *t*-butyl-), 2.77-2.96 (m, 2H, β-CH_2), 4.11-4.39 (m, 1H, α-CH), 4.54 (broad s, 2H, -NH-NH-), 6.80 (d, from AA'BB' pattern of arom., 2H, J ∿ 8-8.5 Hz, $H^{3'}$,$H^{5'}$-arom.), 7.07 (d, ibid 2H, J ∿ 8-8.5 Hz, $H^{2'}$,$H^{6'}$-arom.), 8.05 (broad s, 1H, -NH-CH) ppm.

Z-Pro-OBu^t (16)

A solution of Z-Pro-OH (50 g) in dichloromethane (400 ml) was treated with isobutene (200 ml) and conc. H_2SO_4 (2.0 ml) with stirring in a closed vessel for 2 days at room temperature. The usual work-up procedure gave a yellow crystalline product (54.5 g). Treatment of the crude product in ethanol with activated charcoal for 30 minutes at room temperature gave on filtration of the mixture and evaporation of the filtrate compound 16 as a colourless oil which crystallised spontaneously (51.4 g, 85%); m.p.: 42.5-44°C; $[\alpha]_D^{25}$ = -56.0° (c = 2.0, ethanol); TLC: R_f = 0.80 (A), 0.80 (B), 0.83 (C). Analysis: C, 67.26; H, 7.74; N, 4.67%. $C_{17}H_{23}NO_4$ (305.37) requires C, 66.86; H, 7.59; N, 4.59%. Lit.: Anderson and Callahan (1960): m.p.: 44-45°C; $[\alpha]_D^{25}$ = -52.5° (c = 2.2, ethanol).

Z-Ile-Pro-OBu^t (17)

Compound 16 (19.1 g, 62.6 mmoles) in methanol (150 ml) was subjected to catalytic hydrogenation in the presence of Pd-C (1.0 g) as a catalyst for 45 minutes. The mixture was filtered and concentration of the filtrate *in vacuo* at 20°C

gave H-Pro-OBu^t as a chromatographically homogeneous pale yellow oil. TLC: R_f = 0.46 (A), 0.35 (B), 0.46 (C). To a solution of this oil and Z-Ile-OH (16.6 g, 62.6 mmoles) in DMF (50 ml) was added HOBt (16.9 g, 125 mmoles) in DMF (50 ml), which caused the yellow colour to intensify. The mixture was cooled to -15°C and then treated with DCC (13.5 g, 67.6 mmoles). The solution was kept at -15°C for 1 hour and then a further 20 hours at 0°C. The reaction mixture was filtered, and the filtrate was evaporated *in vacuo* to give a syrup, that was worked-up in ether in the usual manner. Concentration of the resulting solution gave 17 as a pale yellow oil [25.1 g, 95.8%; R_f = 0.80 (A)], which still contained two minor impurities [R_f = 0.71 (A) and R_f = 0.37 (A)]. Chromatography of the crude product on silica gel gave, on development with chloroform containing 2% methanol, chromatographically pure 17 as a thick colourless syrup. $[\alpha]_D^{22}$ = -93.7°(c = 1.5, MeOH); TLC: R_f = 0.80 (B), 0.74 (C). Lit.: Schattenkerk *et al.* (1973).

Bu^t
Z-Tyr-Ile-Pro-OBu^t (18)

Compound 17 (20.75 g, 49.6 mmoles) in methanol (150 ml) was hydrogenated for 1 hour in the presence of palladium on charcoal (0.65 g) as the catalyst. The solution was filtered, and the solvent was evaporated *in vacuo* to give H-Ile-Pro-OBu^t (13.5 g, 47.5 mmoles) as a clear, colourless syrup, which appeared as a single spot on TLC [R_f = 0.46 (C), R_f = 0.27 (B)].

To a solution of 15 (18.3 g, 47.5 mmoles) in dry DMF (350 ml) at -40°C, 2.77 M HCl in ethyl acetate (47.5 ml) was added during 10 minutes with stirring, followed by tert. butyl nitrite (6.84 ml, 59.3 mmoles). The mixture was stirred at -30°C for 30 minutes, whereon a solution of freshly prepared H-Ile-Pro-OBu^t (47.5 mmoles) in DMF (150 ml) was added slowly with stirring. The mixture was neutralized

at -50°C with ethyldiisopropylamine (21.9 ml). The pH was then adjusted to 6-8, and the temperature was allowed to rise to 0°C. Thin layer chromatography of the mixture after 30 minutes revealed that about 80% of the dipeptide had already been acylated. After 16 hours at 0°C the solvent was evaporated *in vacuo* and the residual yellow syrup was dissolved in ethyl acetate (300 ml) and processed as usual to give a pale yellow oil, which was crystallised from ethyl acetate-diisopropyl ether to give 18 (21.2 g, 70%) as white needles; m.p.: 142-143.5°C; $[\alpha]_D^{22}$ = -69.0° (c = 0.5, MeOH); TLC: R_f = 0.80 (A), 0.88 (C). Analysis: C, 67.96; H, 7.95; N, 6.63%. $C_{36}H_{51}N_3O_7$ (637.82) requires C, 67.79; H, 8.06; N, 6.59%.

Bu^t
H-Tyr-Ile-Pro-OBu^t (19)

The catalytic hydrogenation of 18 (8.0 g, 12.5 mmoles) in methanol (160 ml) solution in the presence of palladium on charcoal as the catalyst (0.5 g) was complete within 35 minutes. The resulting solution was filtered and the filtrate concentrated to approximately 15 ml. Dilution with diisopropyl ether gave a cloudy solution, from which a gelatinous precipitate deposited upon storage at 0°C for 15 hours. The precipitate of 19 was collected by filtration and dried to give an analytically pure, white powder (5.52 g, 89%); m.p.: 127-128°C; $[\alpha]_D^{22}$ = -80.4° (c = 1.14, MeOH); TLC: R_f = 0.73 (A), 0.62 (C). Analysis: C, 66.82; H, 8.92; N, 8.45%. $C_{28}H_{45}N_3O_5$ (503.68) requires C, 66.77; H, 9.01; N, 8.34%.

Boc Boc
Z-Lys-Lys-N$_2$*H*$_3$ (20)

The dipeptide ester Z-Lys(Boc)-Lys(Boc)-OMe [5.0 g, 8.03 mmoles; Schwyzer *et al.*, 1961; TLC: R_f = 0.79 (A), 0.86 (C)] was treated with hydrazine hydrate (2.0 ml, 40

mmoles) in methanol (50 ml) at room temperature for 20 hours. The solution was then evaporated to a volume of 10 ml, and water (7 ml) was then added, which induced the product to crystallise. Recrystallisation from methanol-water (1:1) gave the hydrazide 20 (4.5 g, 92%). Two minor impurities (TLC: R_f = 0.70 and 0.74 (A); <2%) could not be removed by recrystallisation. M.p.: 106-110°C; $[\alpha]_D^{22}$ = -18.6° (c = 1.07, MeOH); TLC: R_f = 0.60 (A), 0.72 (C). Analysis: C, 57.82; H, 8.14; N, 13.48%. $C_{30}H_{50}N_6O_8$ (622.76) requires C, 57.85; H, 8.09; N, 13.50%.

Boc Boo Bu^t
Z-Lys-Lys-Tyr-Ile-Pro-OBu^t (21)

The hydrazide 20 (4.35 g, 7.0 mmoles) in dry DMF (70 ml) was cooled to -40°C and then treated sequentially with stirring with 2.77 M HCl in ethyl acetate (7.0 ml) and *t*-butyl nitrite (1.0 ml, 8.75 mmoles). The mixture was then stirred for 30 minutes at -30°C and then treated with a solution of compound 19 (3.52 g, 7.0 mmoles) in DMF (10 ml). The mixture was cooled to -40°C and neutralised to pH 7-8 by addition of ethyl diisopropylamine (3.22 ml, 19.25 mmoles). The reaction was allowed to age for 15 hours at 0°C. The solvents were evaporated *in vacuo* to give a thick syrup, which was dissolved in ethyl acetate and processed in the usual manner. The washed and dried (Na_2SO_4) ethyl acetate solution was evaporated *in vacuo* to give a thick, colourless oil, which crystallised spontaneously. The product was dissolved in hot ethyl acetate (50 ml) and crystallised as small needles on cooling (2.30 g). A second crop (3.92 g) with the same appearance was obtained from methanol; total yield: 6.22 g (80.0%); m.p.: 183.5-185°; $[\alpha]_D^{22}$ = -57.0° (c = 0.63, MeOH); TLC: R_f = 0.84 (A), 0.93 (C). Analysis: C, 63.69; H, 8.34; N, 8.94%. $C_{58}H_{91}N_7O_{13}$ (1094.40) requires C, 63.65; H, 8.38; N, 8.96%.

Msc Msc
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Z-Lys-Lys-Tyr-Ile-Pro-OH (23)

The pentapeptide ester 21 (3.0 g, 2.74 mmoles) was dissolved in 90% aqueous trifluoroacetic acid (30 ml) at -10 C, and the clear solution was kept at room temperature for 1.5 hours. Dropwise addition of this solution to dry ether (250 ml) at 0°C with stirring caused precipitation of the partially protected pentapeptide 22, which was collected by filtration, washed several times with ether, and dried over KOH-pellets *in vacuo*. Yield: 2.67 g (95%, calculated for the di-TFA-salt); TLC: R_f = 0.27 (C), 0.41 (H). The absence of *t*-butyl resonances in the NMR spectrum confirmed that complete deprotection had occurred. The product (2.0g, 1.98 mmoles) was dissolved in water (18 ml) and the pH of the solution was adjusted to 9.7 by addition of ethyl-diisopropylamine (20% solution in DMF). The solution was diluted with DMF (5 ml) and a solution of Msc-ONSu (1.33 g, 5 mmoles) in DMF (5 ml) was then added within 5 minutes, whilst the pH was maintained at 8-8.5 by simultaneous injection of a solution of base in DMF. The reaction mixture, ultimately containing approximately equal volumes of DMF and water, was stirred for a further 10 minutes and then poured into water (120 ml). When necessary the pH was readjusted to approximately 8, and the solution was then extracted with ethyl acetate (3x). The pH was adjusted to 1-2 by addition of 2 M $KHSO_4$ (25 ml), whereon an oil was precipitated. The product was extracted with ethyl acetate (100 ml) and the aqueous phase was then re-extracted with ethyl acetate (3x, 50 ml). The combined extracts were washed with water until neutral and then evaporated to give a syrup. Precipitation from methanol-diisopropyl ether gave pure 23 as the monohydrate (1.91 g, 88.9%); m.p.: ca. 77°C (dec); $[\alpha]_D^{22}$ = -44.0° (c = 0.88, MeOH); TLC: R_f = 0.25 (A), 0.57 (C). Analysis: C, 52.65; H, 6.71; N, 8.98%. $C_{48}H_{71}N_7O_{17}S_2$ (1100.27) requires C, 52.39; H, 6.69; N, 8.91%.

Msc Msc
Z-Lys-Lys-Tyr-Ile-Pro-N_2H_2-Boc (24)

DCC (0.192 g, 0.928 mmoles) was added to a mixture of 23 (0.93 g, 0.835 mmoles), HOBt (0.15 g, 1.08 mmoles) and *t*-butyl carbazate (0.165 g, 1.25 mmoles) in DMF (10 ml), cooled to -15°C. The clear mixture was stirred at this temperature for 30 minutes and then allowed to stand at 0°C for 16 hours. The precipitate of DCU was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. A solution of the residue in butanol-1 was extracted in the usual manner and evaporated to give a syrup, which was redissolved in a mixture of chloroform and methanol (95:5) and applied to a silica gel column (2.5 x 12 cm), prepared in the same solvent mixture. The column was developed until residual dicyclohexylurea had been eluted. The proportion of methanol in the eluent was then raised to 10% and elution continued. Fractions containing the desired product were combined and evaporated to give a syrup, which, on treatment with methanol-diisopropyl ether, gave 24 as a chromatographically pure, amorphous solid (0.839 g, 84%); m.p.: 105-109°C (dec); $[\alpha]_D^{22}$ = -67.3° (c = 0.89, MeOH); TLC: R_f = 0.51 (A), 0.64 (C). Analysis: C, 53.15; H, 6.98; N, 10.45%. $C_{53}H_{81}N_9O_{18}S_2$ (1196.40) requires C, 53.21; H, 6.82; N, 10.54%. Amino acid analysis after total acid hydrolysis: Pro, 1.04; Ile, 0.93; Tyr, 0.97; Lys, 2.05.

Msc Msc
H-Lys-Lys-Tyr-Ile-Pro-N_2H_2-Boc.HOAc (24a)

Hydrogenation of 24 (0.804 g, 0.672 mmoles) in methanol (12 ml) and acetic acid (6 ml) in the presence of palladium on charcoal (0.08 g) as a catalyst for 1 hour gave, on concentration of the filtered reaction mixture *in vacuo*, a colourless foam. A methanol solution (4 ml) of this material was added dropwise to cold ether with stirring. The precipitate of 24a was collected by filtration, washed with

ether and dried *in vacuo* (0.68 g, 90%). TLC: R_f = 0.36 (C).

Boc-Thr-N_2H_3 (27)

t Butyloxycarbonyl threonine, prepared from threonine by the pH stat method of Schnabel (1967) [syrup, 88%; TLC: R_f = 0.70 (C)], was quantitatively converted into the methyl ester [TLC: R_f = 0.74 (C)] by treatment with ethereal diazomethane in ethanol-ether solution. The syrupy ester (30.5 g, 0.131 mole) in methanol (150 ml) was then treated with hydrazine hydrate (19.4 ml, 0.393 mole) at 0°C for 3 days. The solvents were partially evaporated, and the hydrazide, which crystallised in the cold, was collected by filtration and washed with ether. Recrystallisation from ethanol-ether gave needles of 27 (79% yield); m.p.: 127-128°C; $[\alpha]_D^{24}$ = -9.0° (c = 0.99, pyridine); TLC: R_f = 0.51 (C). Analysis: C, 46.21; H, 8.21; N, 18.00%. $C_9H_{19}N_3O_4$ (233.37) requires C, 46.34; H, 8.21; N, 18.01%.
Lit.: Schröder and Gibian (1962): 83.4%; m.p.: 126-128 C; $[\alpha]_D^{24}$ = -9.8° (c = 1, pyridine).

H-Lys(Msc)-OH (25)

The introduction of the methylsulphonylethyloxycarbonyl group at the N^{ε}-position of lysine was performed *via* the copper complex of the amino acid. The pH-stat method of Tesser (1975) was followed, except that the pH was maintained at 10.2 instead of 10.8. The blue copper complex of the product was obtained in 93% yield. Removal of the copper with hydrogen sulfide and recrystallisation from ethanol-water gave pure 25 (71%). M.p.: 222-224°C; $[\alpha]_D^{25}$ = +15.0° (c = 1.04, 1 N HCl); TLC: R_f = 0.17 (C). Analysis: C, 40.55; H, 6.71; N, 9.47%. $C_{10}H_{20}N_2O_6S$ (296.34) requires C, 40.53; H, 6.80; N, 9.45%.
Lit.: Tesser and Balvert-Geers (1975): 78%; m.p.:217-224 C; $[\alpha]_D^{21}$ = +13.5° (c = 1.0, 1 N HCl).

Msc
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H-Lys-OBzl.TosOH (26)

Compound 25 (25.1 g, 84.7 mmoles) and *p*-toluenesulphonic acid monohydrate (102 mmoles) were dissolved in benzyl-alcohol (130 ml). Benzene (400 ml) was added and the mixture was heated to boiling in a Dean-Stark type water separator. Chromatographic analysis revealed that the esterification was complete after 5 hours. Cooling the mixture to room temperature and then addition of ether (500 ml) induced crystallisation of the product. Recrystallisation from methanol-ether gave analytically pure 26 (38.7 g, 82%); m.p.: 94-95.5°C; $[\alpha]_D^{23}$ = -1.5° (c = 1.0, MeOH); TLC: R_f = 0.44 (C). Analysis: C, 51.28; H, 6.06; N, 4.94; S, 11.51%. $C_{24}H_{34}N_2O_9S_2$ (558.67) requires C, 51.59; H, 6.13; N, 5.01; S, 11.48%.

Msc
|
Boc-Thr-Lys-OBzl (28)

A solution of 27 (3.15 g, 13.5 mmoles) in DMF (130 ml) at -30°C was treated with stirring with 2.77 N HCl in ethyl acetate (13.4 ml, 37.1 mmoles) and tert.butyl nitrite (1.86 ml, 16.2 mmoles). The mixture was kept at -30°C for 2.5 hours and then neutralised by adding ethyldiisopropyl-amine (6.39 ml, 37.1 mmoles). Compound 26 (7.54 g, 13.5 mmoles) was then added immediately, followed by an equivalent amount of the tertiary base (2.33 ml, 13.5 mmoles). The apparent pH of the solution, as determined on moist indicator paper, was 7.5. The temperature was allowed to rise to 0°C and the solution maintained at this temperature for 72 hours. DMF was evaporated *in vacuo* to give a syrup, which after dissolution in ethyl acetate followed by the usual work-up procedure, afforded 28, which was crystallised from ethyl acetate-diisopropyl ether. Yield: 6.1 g (77%); m.p.: 95-96°C; $[\alpha]_D^{27}$ = -11.5° (c = 1, ethyl acetate); TLC: R_f = 0.67 (A), 0.75 (C), 0.18 (G). Analysis: C, 53.26; H, 7.01; N, 7.04;

S, 5.46%. $C_{26}H_{41}N_3O_{10}S$ (587.69) requires C, 53.14; H, 7.03; N, 7.15; S, 5.46%.

In preparations of 28, in which only 15 minutes were used for the hydrazide-azide conversion a second reaction product (up to 30%) could be isolated by chromatography on a silica gel column (ethyl acetate). It was crystallised from petroleum ether-ethyl acetate as fine needles; m.p.: 197-198°C; $[\alpha]_D^{27} = -28.5^\circ$ (c = 1.0, EtOAc). The compound was characterised by elemental analysis and by NMR (DMSO-d^6 solution) as N,N'-bis(butyloxycarbonylthreonyl)hydrazine, Boc-Thr-NH-NH-Thr-Boc; it could be distinguished from Boc-Thr-NH , which gives a very similar elemental analysis, by TLC; diacylhydrazide: R_f = 0.79 (C), 0.61 (A); amide: R_f = 0.69 (C), 0.40 (A) . Analysis: C, 49.89; H, 7.74; N, 12.92%. $C_{18}H_{34}N_4O_8$ (434.49) requires C, 49.46; H, 7.89; N, 12.89%. NMR (DMSO-d^6): 1.21 (d, 6H, J ∿ 6.5 Hz, γ-CH_3), 1.39 (s, 18H, *t*-butyl-), 4.50-4.65 (quintet, 2H, J ∿ 6.5 Hz, β-CH), 5.09-5.31 (m, 2H, α-CH), 7.73 (d, 2H, J ∿ 9.5 Hz, -NHCH-), 8.01 (br.s, 2H, -NH-NH-).
Addition of a little D_2O to the DMSO-d^6 solution caused a fast exchange of the hydrazide protons, while the exchange of the urethane NH proton, detectable by the disappearance of the doublet at 7.73 ppm concomitant with the the distorted quartet at 5.20 ppm of the α-CH to a broad doublet, was much slower.

Msc
Z-Gly-Thr-Lys-OBzl (46)

Compound 28 (3.0 g, 5.1 mmoles) was dissolved in 90% aqueous trifluoroacetic acid and kept for 90 minutes at 0°C. The mixture was concentrated *in vacuo* to give a syrup [homogeneous by TLC: R_f = 0.43 (C), 0.23 (A)], which was dissolved in DMF (50 ml). The apparent pH of the solution was raised to 6 by the addition of N-methylmorpholine. Z-Gly-OTcp (1.98 g, 5.1 mmoles; Wolman, 1967) and one equi-

valent of N-methylmorpholine (0.64 ml) were then added. The DMF was evaporated *in vacuo* after a period of 48 hours at room temperature. The residue was dissolved in ethyl acetate and worked-up in the usual way. The washed and dried (Na_2SO_4) organic phase was partially evaporated and ether was added. The crystalline product was collected and recrystallised from ethyl acetate-ether to give compound 46. Yield: 2.89 g (84%); m.p.: 121-123°C; $[\alpha]_D^{27}$ = -23.0° (c = 1.01, MeOH); TLC: R_f = 0.53 (A), 0.70 (C). Analysis: C, 54.89; H, 6.25; N, 8.24; S, 4.81%. $C_{31}H_{42}N_4O_{11}S$ (678.76) requires C, 54.86; H, 6.24; N, 8.25; S, 4.72%.

H-Gly-Thr-Lys(Msc)-OH (47)

Compound 46 (2.9 g, 4.26 mmoles) in methanol (40 ml) was hydrogenated in the presence of palladium on charcoal (0.29 g) as the catalyst for 3 hours. The product, which crystallised during the reaction, was dissolved by adding water (50 ml). The catalyst was then filtered off and the filtrate was evaporated *in vacuo* to dryness. The syrupy residue was dissolved in methanol. Addition of ether precipitated the tripeptide 47 (1.85 g, 96%), which contained 1.5 mole of water per mole, even after drying. M.p.: 177°C (dec); $[\alpha]_D^{27}$ = -25.0° (c = 1.0, water); TLC: R_f = 0.13 (C). Analysis: C, 40.01; H, 6.88; N, 11.52; S, 6.89%. $C_{16}H_{30}N_4O_9S.1\frac{1}{2}H_2O$ (481.52) requires C, 39.91; H, 6.91; N, 11.64; S, 6.66%.

H-Thr-Lys(Msc)-OH (29)

Compound 28 (6.0 g, 10.0 mmoles) was catalytically hydrogenated in methanol (90 ml) for 30 minutes. The catalyst was filtered off and the filtrate was evaporated to give Boc-Thr-Lys(Msc)-OH as a clear, colourless syrup, [TLC: R_f = 0.52 (C)], which was dissolved in 90% aqueous trifluoroacetic acid, previously cooled to 0°C. The solution

was kept at this temperature for 3 hours. On slow addition of the mixture to dry ether(1000 ml), with stirring, 29 precipitated. The ether was decanted, and the precipitate was treated once more with dry ether (300 ml), filtered rapidly, washed with ether, and dried *in vacuo* (KOH).

A hygroscopic white powder (29), behaving as a single compound on TLC [R_f = 0.17 (C)], was obtained in a yield of 86%.

Trt-Gly-ONSu (30)

The method of Stelakatos *et al.* (1959) was used to prepare Trt-Gly-OH, except that the trityl chloride was used in DMF-solution. The trityl compound was obtained in a modest yield (29%) (lit.: 48%); m.p.: 161-162°C; TLC: R_f = 0.46 (A). The succinimidyl ester 30 was subsequently prepared according to Anderson *et al.* (1964) in 88% yield; m.p.: 138-139°C; TLC: R_f = 0.87 (A). Lit.: 83%; m.p.: 145.5-146.5°C.

Trt-Gly-Thr-Lys(Msc)-OH (31)

A solution of compound 29 (4.0 g, 7.82 mmoles) in ice-cold DMF (20 ml) was treated with stirring with two equivalents of N-methylmorpholine (0.90 g), followed by 3.3 g (8.0 mmoles) of 30 in DMF(10 ml). The apparent pH of the solution was raised from 4.5 to about 6.5 by addition of N-ethylmorpholine. After 16 hours at 0°C TLC indicated complete reaction of 29. The mixture was poured into water (230 ml) and the pH was adjusted to 8.5 with 2 M $KHCO_3$. The aqueous solution was extracted with ethyl acetate (4 x 50 ml) and then acidified to pH 1.5-2 by addition of 2 M $KHSO_4$. The turbid solution was extracted with ethyl acetate (4 x 50 ml) and the combined ethyl acetate extracts were washed successively with water and saturated sodium chloride solution, and then dried (Na_2SO_4). The solvent was then evaporated to small volume and compound 31 was precipitated as a white

amorphous powder by addition of diisopropyl ether (94%); m.p.: 78°C (dec); $[\alpha]_D^{22}$ =+10.2° (c = 1.33, MeOH); TLC: R_f = 0.06 (A), 0.50 (B), 0.60 (E), 0.62 (E). Analysis: C, 58.82; H, 6.70; N, 7.81%. $C_{35}H_{44}N_4O_9S.1\ H_2O$ (714.82) requires C, 58.81; H, 6.49; N, 7.84%.

Msc-Met-N_2H_2-Boc (39)

Pyridine (2.31 ml, 36.3 mmoles) was added to a freshly prepared solution of Msc-Met-OH (Tesser and Balvert-Geers, 1975) in THF (100 ml). The mixture was then treated, at -15°C and with stirring, with isobutyl chloroformate (3.6 ml, 27.5 mmoles) for 10 minutes. During this time pyridine-HCl precipitated from the mixture. *t*-Butyl carbazate (3.83 g, 29 mmoles) was added to the solution of the mixed anhydride and the mixture was maintained at 0°C for 18 hours. The syrup that was yielded after filtration of the mixture and evaporation of the filtrate, was dissolved in ethyl acetate and worked-up in the usual manner. Crystallisation from ethyl acetate-petroleum ether afforded 39 (7.6 g, 63%); m.p.: 105.5-106°C; $[\alpha]_D^{27}$ = -29.5° (c = 1.0, MeOH); TLC: R_f = 0.56 (A), 0.71 (C). Analysis: C, 40.79; H, 6.46; N, 10.17; S, 15.52%. $C_{14}H_{27}N_3O_7S_2$ (413.51) requires C, 40.66; H, 6.58; N, 10.16; S, 15.52%.

When the mixed anhydride coupling was applied to the dicyclohexylamine salt of Msc-Met-OH, N-isobutyloxycarbonyl-N'-tert.butyloxycarbonylhydrazide appeared to be formed as the main coupling product (NMR). In the procedure described above only trace amounts of this product were detectable.

H-Met-N_2H_2-Boc (33)

Compound 39 (3.0 g, 7.25 mmoles) was dissolved in a mixture of peroxide-free dioxan (60 ml) and methanol (20 ml). To the vigorously stirred solution 4 N NaOH (5.4 ml, 21.6 mmoles) was rapidly added, followed after 20 seconds by acetic acid (1.5 ml) to restore neutrality. The solvents were evaporated and the residue was dissolved in water (80 ml).

The pH was lowered to 2-3 with 2 M $KHSO_4$, and the solution was extracted with ethyl acetate. The pH was then re-adjusted to 10 with 4 N NaOH, and the solution was further extracted several times with ethyl acetate. The combined organic layers were washed with water, dried (Na_2SO_4), and concentrated to give compound 33 as a colourless syrup (78% yield). It was homogeneous by TLC [R_f = 0.25 (A), 0.58 (C)]; the sulfoxide derivative, obtained by addition of a drop of 3% H_2O_2 to 0.2 ml of a 1% solution of 33 in MeOH, had R_f = 0.37(C). Lit.: Moroder *et al.* (1977); yield: 60.5%; m.p.: 73°C; $[\alpha]_D^{25}$ = +15.5° (c = 1.015, MeOH).

Storage of the free amino compound in the refrigerator for prolonged periods caused deterioration as shown by TLC. Compound 33 was prepared freshly when required.

Trt-Gly-Thr-Lys(Msc)-Met-N_2H_2-Boc (34)

A solution of 31 (2.3 g, 3.22 mmoles) in DMF (10 ml) was successively treated at -10° with compound 33 (0.92 g, 3.5 mmoles) in DMF (10 ml), followed by HOBt (0.87 g, 6.4 mmoles) and DCC (0.68 g, 3.3 mmoles). The mixture was stirred for 30 minutes and then left at 0°C for 15 hours. Filtration and evaporation of the filtrate gave a pink coloured foam, which was dissolved in ethyl acetate (60 ml) and processed as usual. The washed and dried ethyl acetate solution was concentrated to a small volume and added dropwise to diisopropyl ether to precipitate the crude product. A second precipitation from the same solvents gave 34 as a slightly coloured powder (2.80 g, 90.6%). The coloured impurity could be removed by chromatography on a silica gel column (cm) with chloroform-methanol (95:5) as the eluent. In this way 630 mg of the crude product gave 510 mg (81%) of chromatographically homogeneous 34. M.p.: 100-108°C (dec); $[\alpha]_D^{22}$ = -21.2° (c = 0.947, MeOH); TLC: R_f = 0.64 (A), 0.69 (B), 0.75 (C). Analysis: C, 57.11; H, 6.59; N, 10.30%. $C_{45}H_{63}N_7O_{11}S_2$ (942.17) requires C, 57.37; H, 6.74; N, 10.41%.

Msc
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H-Gly-Thr-Lys-Met-N_2H_2-Boc.HCl (34a)

The tetrapeptide derivative 34 (100 mg, 0.104 mmoles) was dissolved in 90% aqueous trifluoroethanol; the pH of this solution was 6.1 (pH-meter). The trityl function was cleaved by addition of 0.1 N HCl in 90% aqueous trifluoroethanol in 100 µl portions, which lowered the pH to 3.5-4. During the course of this treatment triphenylcarbinol crystallised from the mixture. When 920 µl of the HCl-solution had been consumed, the pH did not rise further (theoretical amount: 1000 µl). The mixture was then diluted with diisopropyl ether, which dissolved the triphenylcarbinol. The resulting clear solution was then slowly added to diisopropyl ether (30 ml) with stirring. The white precipitate was collected by centrifugation, washed three times with diisopropyl ether, and dried *in vacuo* (KOH). Yield: 90%; m.p.: >117° (dec); $[\alpha]_D^{22}$ = -38.8° (c = 0.86, MeOH). Amino acid analysis following complete acid hydrolysis: Thr, 0.98; Gly, 1.02; Met, 0.88; Lys, 1.00.

Msc
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Trt-Gly-Thr-Lys-N_2H_2-Boc (32)

The trityl tripeptide 31 (0.50 g, 0.70 mmoles) in DMF (3 ml) maintained at -10°C was treated successively with *t*-butyl carbazate (0.12 g, 0.91 mmoles), HOBt (0.114 g, 0.84 mmoles) and DCC (0.159 g, 0.77 mmoles). The mixture was stirred at -10°C for 1 hour and then stored in a refrigerator for approximately 15 hours. The dicyclohexylurea that was formed, was filtered off, and the filtrate was diluted with ethyl acetate (30 ml). The organic solution was washed successively with water, 2 M $KHSO_4$, 2 M $KHCO_3$, water and saturated sodium chloride solution, and then dried (Na_2SO_4). Evaporation to a small volume (~4 ml) followed by addition of diisopropyl ether precipitated compound 32 (0.482 g, 83%); m.p.: ca. 75° (dec); $[\alpha]_D^{22}$ = -8.3° (c = 1.12, MeOH);

TLC: R_f = 0.71 (A), 0.75 (C). Analysis: C, 58.04; H, 6.65; N, 10.02%. $C_{40}H_{55}N_6O_{10}S.H_2O$ requires C, 57.96; H, 6.81; N, 10.14%.

Msc
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H-Gly-Thr-Lys-N_2H_2-Boc.HCl (32a)

This compound was prepared from derivative 32 by the detritylation procedure described for compound 34a. Precipitation of the crude product with diisopropyl ether gave a syrup, which solidified when triturated with ether or ethyl acetate to give a white, hygroscopic solid (94%); m.p.: >92°C (foaming); $[\alpha]_D^{20}$ = -27.5° (c = 1.0, MeOH); TLC: R_f = 0.31 (C).

Msc Msc
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Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-N_2H_2-Boc (35)

A solution of 14a (0.624 g, 0.600 mmoles) in dry DMF (10 ml) was cooled at -35°C, treated with stirring with 2.23 N HCl solution (0.74 ml, 1.65 mmoles), and then immediately supplied with *t*-butyl nitrite (0.89 ml, 10% solution in DMF, 0.75 mmoles). The mixture was stirred at -15°C for 20 minutes and then recooled to approximately -35°C. Ethyldiisopropylamine (10% in DMF, 0.39 ml, 2.25 mmoles) was added and the resulting neutral solution of the azide was thoroughly mixed with a cold solution of 24a (0.614 g, 0.547 mmoles) in DMF (3 ml). The temperature was allowed to rise to 0°C, and the clear solution was then left at the same temperature for 16 hours. The oil that was obtained on evaporation of the solvents *in vacuo* was dissolved in the upper phase of the solvent system butanol-1-acetic acid-water (15:1.5:13.5) and subjected to counter current distribution in this system for a total of 93 transfers. A trace of the amino component 24a and a second, more polar impurity could be separated in this manner. The chromatographically homogeneous material, that was present in tubes 87-92 (10 ml

per tube), was combined and the solvents were evaporated *in vacuo*. The residual gum was dissolved in a small amount of methanol, and addition of diisopropyl ether precipitated compound 35 (0.78 g, 73%); m.p.: ∿127°C (dec), foaming at ca. 152°C; $[\alpha]_D^{22}$ = -67.5° (c = 0.1, MeOH); TLC: R_f = 0.40 (C). Amino acid analysis after complete acid hydrolysis: Asp, 0.98; Glu, 1.96; Pro, 2.11; Ile, 0.95; Leu, 1.00; Tyr, 1.96; Lys, 2.03.

Msc Msc
Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-N_2H_3.TFA (36)

Compound 35 (0.103 g, 0.052 mmoles) was dissolved in 90% aqueous trifluoroacetic acid at -10°C with stirring. A clear solution was obtained after 10 minutes, which was then kept at room temperature for a further 30 minutes. The product was isolated by precipitation with ether and dried *in vacuo* (KOH). Yield: 96.6 mg (93%); TLC: R_f = 0.25 (C).

Msc Msc *Msc*
Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-Thr-Lys-Met-N_2H_2-Boc (37)

Compound 36 (150 mg, 0.076 mmoles) in dry DMF (1.0 ml) at -30°C was treated with stirring with 1.3 N HCl in ethyl acetate (0.150 ml, 0.195 mmoles) and tert-butyl nitrite (109 µl of a 10% solution in DMF, 0.095 mmoles). The solution was stirred at -15°C for 20 minutes and then recooled to -30°C whereon ethyldiisopropylamine (46 µl, 0.271 mmoles) was added, thereby raising the apparent pH of the solution (moist indicator paper) from 1.5 to approximately 6. A chilled solution of 34a (67 mg, 0.091 mmoles) in DMF (250 µl) was then added to the above mixture, immediately followed by ethyldiisopropylamine (28.6 µl, 0.167 mmoles). The pale yellow solution (pH 6-6.5) was stored at 0°C for 48 hours. The reaction mixture was then diluted with DMF (3 ml), and applied to a Sephadex LH-20 column (3.5 x 120 cm), which was

equilibrated and eluted with DMF. The high molecular weight fraction gave the product 37 on subsequent evaporation and precipitation from ether, as an amorphous powder. Yield: 175 mg (90.4%); TLC: R_f = 0.34 (C), 0.55 (H). The product contained a small amount (<5%) of a compound with the mobility of the methionine-sulfoxide analogue of 37 [TLC: R_f = 0.21 (C)]. Counter current distribution in the same solvent system as described for compound 35, completely removed the impurity and gave chromatographically pure 37 in 73.8% overall yield (143 mg); $[\alpha]_D^{22}$ = -40.3° (c = 1.18, MeOH). Amino acid analysis following total acid hydrolysis: Asp, 1.00; Thr, 0.96; Glu, 2.08; Pro, 2.05; Gly, 1.03; Met, 0.92; Ile, 0.95; Leu, 1.02; Tyr, 1.94; NH_3, 1.04; Lys, 3.02.

Msc Msc Msc
Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-Thr-Lys-Met-N_2H_3.TFA (VI)

Treatment of 37 (65 mg) with 90% aqueous trifluoroacetic acid as described for the preparation of 36, gave VI as an amorphous powder (61 mg, 93.5%); $[\alpha]_D^{22}$ = -34.5° (c = 1.00, DMF); TLC: R_f = 0.19 (C), 0.45 (H).

The purity of the pentadecapeptide was established further by chromatography of the completely deprotected peptide. Compound VI (30 mg) was dissolved in DMF-methanol (3:1, 2.25 ml) and 4 M NaOH solution (150 µl) was then added with vigorous agitation (Vortex), after 30 seconds followed by acetic acid (200 µl). Chromatography of the resulting clear solution on Sephadex G-15, using 5% aqueous acetic acid as eluent, and lyophilisation of the eluate yielded the unprotected peptide-hydrazide 66-80. The compound was apparently homogeneous by TLC [R_f = 0.30 (H)] and by cellogel chromatography, carried out as described by Von Arx and Faupel (1976,1977),using a buffer of pH 7.8 (0.025 M borax, 0.05 M KH_2PO_4: R_f-cytochrome c = 0.86) and a buffer of pH 4.65 (0.1 M HOAc, 0.1 M NaOAc) as developers. Detection was by the Barton-reagent.

Amino acid analyses of VI (A) and the deprotected peptide (B) after reaction with fluorodinitrobenzene (see chapter III) and complete acid hydrolysis: Asp, 1.01 (A), 1.00 (B); Thr, 1.00 (A), 0.94 (B); Glu, 2.11 (A), 1.11 (B); Pro, n.d. (A), n.d. (B); Gly, 1.09 (A), 1.05 (B), Met, 0.77 (A), 0.74 (B); Ile, 0.96 (A), 0.93 (B); Leu, 0.99 (A), 1.02 (B); Tyr, 0.14 (A), 0.20 (B); NH_3, 1.44 (A), 1.58 (B), Lys, 3.19 (A), 0.11 (B).

Msc Msc Msc
Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-Thr-Lys-
N_2H_2-Boc (38)

A solution of 36 (130 mg, 0.066 mmoles) in DMF (20 ml) was treated at -30°C with stirring with 4.06 N HCl in ethyl acetate (111 µl, 0.455 mmoles) and tert-butyl nitrite (94 µl of a 10% solution in DMF, 0.082 mmoles). The mixture was kept at -15°C for 15 minutes, cooled to -40° and neutralised with ethyldiisopropylamine (103 µl, 0.600 mmoles) and then treated with 32a (48 mg, 0.079 mmoles) in chilled DMF (0.5 ml). The reaction mixture, the pH of which was 6.7, was stirred at -10°C for 1 hour and then kept at 0°C for 20 hours. Pouring the reaction mixture into diisopropyl ether gave a gum, which was partitioned between butanol-1 (5 ml) and 10% aqueous acetic acid (5 ml). The aqueous phase was separated and the organic layer was extracted a further two times with the same solvent. This extraction procedure removed completely the excess of 32a. The butanol extract was evaporated *in vacuo*, and the resulting foam, on precipitation from methanol and ether, gave the crude tetradecapeptide 38 (120 mg, 75%). TLC showed that the product [R_f = 0.37 (C)] contained two impurities [R_f = 0.26 and R_f = 0.43 (C)], which could not be removed by gel filtration on a Sephadex LH-20 column, eluted with 90% aqueous DMF. Treatment of a sample of crude 38 with 90% aqueous trifluoroacetic acid did not affect the chromatographic mobility of the side products, which evidently did not contain a

t-butyloxycarbonyl function. Further purification appeared more convenient with the partially deprotected peptide hydrazide V.

Msc Msc Msc
Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-Thr-Lys-
N_2H_3.TFA (V)

The above product was treated with 90% aqueous trifluoroacetic acid as described for compound 36 and was then subjected to counter current distribution in the system butanol-1-acetic acid-water (4:1:5) for 170 transfers. A chromatographically homogeneous product [TLC: R_f = 0.10 (C)] was obtained (K = 0.48, ν_{max} = 55) as an amorphous powder by precipitation with ether, in an overall yield of 60% (96 mg); $[\alpha]_D^{22}$ = -32.6° (c = 1.06, MeOH); TLC: R_f = 0.35 (H). Amino acid analysis after total acid hydrolysis: Asp, 1.03; Thr, 0.90; Glu, 2.15; Pro, 1.98; Gly, 1.05; Ile, 0.96; Leu, 0.99; Tyr, 1.96; NH , 1.30; Lys, 2.87.

Z-Gly-Thr-OMe (40)

A solution of Z-Gly-OPfp (7.6 g, 20.2 mmoles) (prepared by the method of Kisfaludy *et al.* (1974); m.p. 81.5-83°C; lit. 86-87°C) in diethyl ether (70 ml) was added at room temperature to a solution of H-Thr-OMe (2.88 g, 21.6 mmoles) in tetrahydrofuran (70 ml). A product crystallised during the ensueing exothermic reaction, and after 15 minutes ether (100 ml) was added, and the mixture was allowed to cool to 0°C to complete crystallisation. Pure 40 was collected by filtration, washed with ether, and dried *in vacuo* (5.50 g, 84.8%); m.p.: 101.5-102°C; $[\alpha]_D^{22}$ = -5.2° (c = 1.12, MeOH); TLC: R_f = 0.69 (B), 0.74 (C). Analysis: C, 55.36; H, 6.24; N, 8.68%. $C_{15}H_{20}O_6N_2$ (324.33) requires C, 55.55; H, 6.22; N, 8.64%.
Lit.: Moroder *et al.* (1973): 49%; m.p.: 90-91°C; $[\alpha]_D$ = -3.4° (c = 1.03, MeOH).

Z-Gly-Thr-N_2H_3 (41)

The dipeptide ester 40 (10.0 g, 30.8 mmoles) was dissolved in methanol (90 ml) and treated with hydrazine hydrate (4.17 ml, 86 mmoles). Storage for 18 hours at room temperature gave the product which was collected by filtration. Addition of water to the filtrate gave a second crop. Recrystallisation from water afforded the pure hydrazide 41 (8.4 g, 84.1%); m.p.: 173-175°C; $[\alpha]_D^{22}$ = +6.5° (c = 1.3, DMF); TLC: R_f = 0.43 (C). Analysis: C, 52.02; H, 6.31; N, 17.15%. $C_{14}H_{20}O_5N_4$ (324.34) requires C, 51.85; H, 6.22; N, 17.27%.
Lit.: Moroder *et al.* (1973): 81%; m.p.: 176-178°C; $[\alpha]_D$ = +8.1° (c = 0.805, DMF).

Ac
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Z-Lys-N_2H_2-Boc (45)

A solution of Z-Lys(Ac)-OH (9.33 g, 29.0 mmoles), prepared as described in Chapter VI, in ethyl acetate (200 ml) maintained at 0° was treated successively with *t*-butyl carbazate (4.1 g, 33.6 mmoles, HOBt (4.7 g, 35 mmoles) and DCC (6.18 g, 30 mmoles). The mixture was stirred at 0° for 1 h and then left at room temperature for 16 h. The dicyclohexylurea was filtered off and the filtrate was partly evaporated. On storage at 0° the product crystallized slowly from the solution. The product was collected by filtration and recrystallized twice from propanol-2 to give analytically pure 45 (9.1 g, 72%); m.p.: 118-120°; $[\alpha]_D^{23}$ = -29.5° (c = 0.92, MeOH); TLC: R_f = 0.83 (B), 0.76 (C). Analysis: C, 57.66; H, 7.39; N, 12.71%. $C_{21}H_{32}O_6N_4$ (436.59) requires C, 57.76; H, 7.39; N, 12.83%.

Ac
|
Z-Gly-Thr-Lys-N_2H_2-Boc (44)

The hydrazide 41 (0.7 g, 2.16 mmoles) in DMF (20 ml) was cooled to -30° and then treated sequentially, with stirring, with 3.9 N HCl in ethyl acetate (1.52 ml, 5.93 mmoles) and *t*-butyl nitrite (0.31 ml, 2.7 mmoles). The

mixture was stirred for 10 minutes at -20° and then neutralised at -40° with ethyldiisopropylamine (1.02 ml). The solution of the azide was combined with H-Lys(Ac)-N_2H_2-Boc, freshly prepared by hydrogenation of 45 (1.00 g, 2.30 mmoles) in methanol, in DMF (5 ml) and then left to stand at 0° for 48 hours. The solvents were evaporated and the residue was dissolved in water-saturated butanol-1. The organic solution was washed with 1 N $KHSO_4$, 1 N $KHCO_3$ and water. The syrupy residue that was obtained on evaporating the solvents, was dissolved in warm ethyl acetate. On cooling 44 crystallised in a 79% yield (1.01 g); m.p.: 153-157 ; $[\alpha]_D^{20}$ = -28.7° (c = 1.0, MeOH); TLC: R_f = 0.39 (B), 0.66 (C). Analysis: C, 53.57; H, 7.12; N, 13.97%. $C_{27}H_{42}N_6O_9.\frac{1}{2}\ H_2O$ requires C, 53.72; H, 7.35; N, 13.92%.

Msc
|
Z-Lys-N_2H_2-Boc (42)

Z-Lys(Msc)-OH (2.5 g, 5.79 mmoles; Tesser and Balvert-Geers, 1975) in ethyl acetate (40 ml) was treated successively at 0° with *t*-butyl carbazate (0.8 g, 6.0 mmoles), HOBt (0.77 g, 5.8 mmoles) and DCC (1.19 g, 5.79 mmoles). The mixture was stirred for 1 hour at 0° and then kept at room temperature for 15 hours. Precipitated dicyclohexylurea was removed by filtration and the filtrate was worked-up in the usual manner. The resulting ethyl acetate solution was dried (Na_2SO_4) and concentrated *in vacuo* to give 42 (2.9 g, 91%) as a thick, colourless oil [TLC: R_f = 0.66 (B), 0.67 (C)], which still contained a trace of dicyclohexylurea. The product could not be crystallized.

Msc
|
Z-Gly-Thr-Lys-N_2H_2-Boc (43)

Hydrogenation of 42 (2.9 g, 5.3 mmoles) in methanol solution gave, on filtration of the catalyst and evaporation of the filtrate, H-Lys(Msc)-N_2H_2-Boc as a foam [TLC: R_f = 0.11 (B), 0.17 (C)]. This derivative of lysine was treated

in DMF (10 ml) at 0° with the azide obtained from 41 (1.79 g, 5.39 mmoles), as previously described in the preparation of 44. The reaction was allowed to proceed for 24 hours at 0°. Solvents were evaporated *in vacuo* to give a syrup, which was subsequently dissolved in butanol-1 and processed in the usual way. Concentration *in vacuo* gave a sticky residue, which was dissolved in methanol. On prolonged cooling the product crystallised. The crystallisation from methanol was repeated twice and gave 1.7 g (45%) of 43, which did not have a characteristic melting point (120-140°); $[\alpha]_D^{20}$ = -24.3° (c = 1.63; MeOH); TLC: R_f = 0.53 (B), 0.84 (C). Analysis: C, 49.00; H, 6.74; N, 11.54%. $C_{29}H_{46}N_6O_{12}S.1\ CH_3OH$ (734.82) requires C, 49.02; H, 6.86; N, 11.44%.

CHAPTER V

SEMISYNTHESIS OF HSE^{65}-CYTOCHROME C FROM THREE FRAGMENTS

The methods described in Chapters II, III and V have been published:

-Boon, P.J., Tesser, G.I. and Nivard, R.J.F. (1978). In: 'Semisynthetic Peptides and Proteins' (Offord, R.E. and DiBello, C., eds.) pp 115-126. Academic Press, New York.

-Boon, P.J., Tesser, G.I. and Nivard, R.J.F. (1979) Proc. Natl. Acad. Sci. USA *76*, 61-65.

*SEMISYNTHESIS OF HSE^{65}-CYTOCHROME C FROM THREE FRAGMENTS**

5.1. Introduction

In preceding chapters the methods used to obtain the 'natural' fragment 1-65-lactone (IX, Chapter II) and its recombination with the protected 'natural' sequence 66-104, the preparation of the synthetic sequences 66-79 (V) and 66-80 (VI, Chapter IV) and of the N^{ε}-protected 'natural' sequence 81-104 (II, Chapter III) have been described. The semisynthesis of the sequence 66-104 (VIII) of cytochrome c is the remaining problem.

Two routes were examined (Figure 1), which are described in this chapter. The originally envisaged, direct approach, route B, proved to be troublesome, presumably due to some intramolecular side reaction

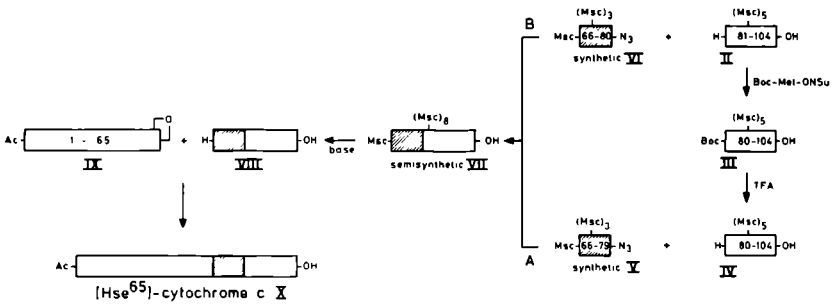

Fig 1 Strategy for the semisynthesis of Hse^{65}-cytochrome c

of the azide VI. Under optimal conditions fragment VIII was obtained in moderate yield (20-37%). Route A, which differs from route B by the transposition of Met^{80} from the azide to the amino component, gave VIII in a satisfactory yield of 60-65%.

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*In this chapter the peptide-size nomenclature proposed by M. Bodanszky at the Fifth American Peptide Symposium (San Diego, 1977) has been used. Greek prefixes are retained only for di- to decapeptides. For peptides with more than 10 amino acid residues arabic numerals have been used.

Semisynthetic Hse^{65}-cytochrome c (X) was obtained from the semisynthetic 39-peptide VIII, originating from route A as well as route B, by coupling with the native 1-65-lactone. Compound X appeared to be indistinguishable from the Hse^{65}-cytochrome c, produced from the natural sequences 1-65 and 66-104, with respect to biophysical and functional properties.

5.2. Properties of the 15-peptide azide VI

The acylation of the Msc-protected cytochrome c fragment 81-104 (II) with the synthetic 66-80 peptide azide VI to produce the semisynthetic sequence 66-104 of cytochrome c proved to be difficult.

VI Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro-
66 70
-Gly-Thr-Lys(Msc)-Met-N_3
80

II H-Ile-Phe-Ala-Gly-Ile-Lys(Msc)-Lys(Msc)-Lys(Msc)-Thr-Glu-
81 90
-Arg-Glu-Asp-Leu-Ile-Ala-Tyr-Leu-Lys(Msc)-Lys(Msc)-Ala-Thr-
100
-Asn-Glu-OH
104

In fact, the initial attempts failed completely. This result was not surprising at the time, since it confirmed the results of Borin *et al* (1976), who attempted a similar condensation with fragments 66-80 and 81-104, in which the amino functions had been protected by trifluoroacetyl groups. These workers reported that in model reactions of Z-Lys(TFA)-Met-N_3 with H-Ile-Phe-Ala-NHNH-Boc or H-Gly-Phe-Ala-NHNH-Boc satisfactory yields (60%) of the expected pentapeptides were obtained, demonstrating that the side chains of the terminal residues, Met and Ile, did not interfere with the reaction.

The main reason of the failure to couple VI and II has to be ascribed to the deviating reactivity of the 15-peptide azide VI. This was concluded from the similar low yields obtained in attempts to acylate H-Trp-Gly-OMe with VI. The dipeptide was chosen as a model amino compound, since the amino function of an N-terminal tryptophylpeptide is known to be a

a good nucleophile, whereas the presence of tryptophan in the reaction product enables specific detection by the Ehrlich reagent after thin layer chromatography.

The formation of the azide VI, on treatment of the hydrazide of VI with *t*-butyl nitrite by the method of Honzl and Rudinger (1961) was unquestionable since the IR-spectrum of the reaction mixture showed the characteristic absorption at 2130 cm^{-1}. Moreover, monitoring of the azide by IR for 16 hours showed that the azide was not prone to isocyanate formation; no new absorption band at ca 2230 cm^{-1} developed during this periode. Evidence for the possible cause of the apparent unreactivity of the azide came from a comparison of model reactions between several azides related to VI and H-Trp-Gly-OMe or H-Val-Tyr-Val-His-Pro--Phe-OBu^t as the amino component. The hexapeptide (angiotensine II hexapeptide) served as a sterically more hindered nucleophile. The results are presented in Table 1.

Table 1

Azide	A		B	
	2 h	16 h	2 h	16 h
a: VI: Msc-Glu-Tyr-Leu-Glu-Asn-Pro--Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro--Gly-Thr-Lys(Msc)-Met-N_3	10-40%	10-40%	10-30%	10-30%
b: Msc-Gly-Thr-Lys(Msc)-Met-N_3	30-50%	50-60%	20-30%	30-40%
c: Msc-Gly-But^{t}-Thr-Lys(Msc)-Met-N_3	60-70%	>90%	40-60%	>90%
d: Msc-Gly-Thr-Lys(Msc)-N_3				
e: Msc - Met-N_3				
f: V : Msc-Glu-Tyr-Leu-Glu-Asn-Pro--Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro--Gly-Thr-Lys(Msc)-N_3				n.d.

Product yields from reactions in which equivalent amounts of the azide and amino component, H-Trp-Gly-OMe (A) or H-Val-Tyr-Val-His-Pro-Phe-OBu^t (B), were allowed to react in DMF at concentrations of 10-20 mM. Yields were estimated, after 2 and 16 hours, from intensities of products on TLC plates when viewed under UV-light, and after detection with Barton, Ehrlich and Pauly reagents, when appropriate.

It appeared that the tetrapeptide b, having the same C-terminal sequence as VI also gave relatively low yields. However, when the threonyl residue in the tetrapeptide was protected at the hydroxyl group (c), or its position relative to the azide group was varied (d, f) near quantitative yields were obtained. This observation suggested that the position of the threonyl residue relative to the azide function was the determining factor.

In a further series of model reactions with VI and H-Trp-Gly-OMe, in which the experimental conditions during azide formation and subsequent coupling were varied, it was revealed that the only condition that influenced the final coupling yield was the concentration of the reactants: greatest yields were obtained at concentrations of 10-20 mM. This explains the complete failure of the first attempts to prepare VIII, in which the concentration of the azide VI did not exceed 2 mM, because of the solubility properties of the amino component II (see section 5.3).

These observations suggested that some unimolecular reaction interfered with the bimolecular coupling reaction. Although the exact nature of the side reaction remains unclear, the involvement of the free hydroxyl function of Thr^{78} seems to be indicated. Amide formation, which can occur during the conversion of the hydrazide into the azide, cannot be an important side reaction, since the presence of the azide was firmly established. Furthermore, no concentration dependence of the subsequent coupling would then be expected. Enhanced hydrolysis of the azide, catalysed by the hydroxyl function of Thr^{78}, would be in accord with the experimental data obtained.

Related observations of low reactivity of peptides containing hydroxy amino acids in the C-terminal part have been reported in the literature: in synthetic studies on ribonuclease T_1 (Waki *et al*, 1968; Beacham *et al*, 1971) acylations with Z-Ser-Ser-Ser-N_3 appeared unsuccessful. Waki *et al* (1968) reported that amide formation occurred during the preparation of the azide. Z-Ser-Ser-N_3, on the other hand, behaved normally. In studies on the synthesis of glucagon, Wünsch *et al* (1968) found that Boc-His-Ser--Gln-Gly-Thr-Phe-Thr-Ser-NH_3 (sequence 1-8) failed to acylate (yields <3%) the 9-23 sequence of the hormone and therefore changed their strategy. The unusually slow rate of saponification of Z-Ser-Tyr-Ser-OMe (Guttmann and Boissonnas, 1958) provides a further example of an unreactive sequence.

The common feature in all these instances is the occurrence of a free hydroxyl function at an almost fixed distance from the reaction centre.

The model reactions suggested two, more promising methods for the semisynthesis of VII:

1. the normal reactivity of the 14-peptide azide V (Table 1, entry f) could be explored following the strategy indicated as route A in Figure 1
2. route B could be attempted again in a solvent in which II is more soluble (up to concentrations of 10-20 mM).

These possibilities will be described in the following sections.

5.3. N^{α}-Boc, $N^{\varepsilon 86\text{-}88,99,100}$-*penta-Msc-cytochrome c-(80-104)-pentacosapeptide (III)*

For the extension of the 24-peptide derivative II with a methionyl residue the use of an active ester of Boc-Met-OH was the method of choice, because the Boc-group can be selectively removed from the product III by trifluoroacetic acid treatment, without interference with Msc-groups.

An advantage of the use of active esters is that they can be supplied in a large excess over the amino component in order to approach quantitative acylation. It must be realized, however, that in the case of minimal side chain protection of a large amino component this implies the risk of side reactions at other nucleophilic side chain functions, such as the hydroxyl groups of Ser, Tyr and Thr and even the carboxyl groups of Asp and Glu. This is particularly true when the amino component contains histidyl residues (Bodanszky *et al*, 1977; Bodanszky and Fagan, 1977).

Bodanszky has shown that addition of an equivalent amount of N-hydroxybenzotriazole (HOBt) reduces the extent of O-acylation, but injudicious use of this additive must be avoided since in the absence of an imidazole side chain it seems to enhance O-acylation (Klausner and Chorev, 1975) rather than to decrease it. Moreover, HOBt catalyses the racimisation of the C-terminal amino acid residue of an amino component on acylation with amino acid p-nitrophenyl- and especially pentachlorophenyl esters (Sakakibara, 1977). Mixed anhydride formation has been suggested as a possible mechanism of this racimization (Natarajan and

Bodanszky, 1976).

The 24-peptide derivative II does not contain His or Ser. Therefore, HOBt has not been added. Unwanted O-acylation at the only tyrosyl or two threonyl residues was never observed under these conditions.

Two methods of extending the fragment 81-104 by a methionine residue have been employed. In the first route the acylation was done in aqueous DMF with the N-hydroxysuccinimide ester of Boc-Met-OH: 10 equivalents of the reagent were used, while the pH of the reaction mixture was maintained at 8-8.5 by the addition of ethyldiisopropylamine. These conditions closely resemble those developed for the introduction of Msc-groups in cytochrome c (cf Chapter III). The acylation proved to be rapid, but complete acylation was never observed. This is presumably due to the solubility properties of derivatives II and III.

The starting compound II, used as a lyophilisate, did not readily dissolve in neat DMF, DMSO or HMPT, even after neutrilization of the acidic function with ethyldiisopropylamine. Addition of water up to approximately 10-15% of the total volume gave immediately a clear solution, which, however, invariably became viscous or gelatinous within a short time. Only dilution to 0.5-1% solutions of II gave sufficiently thin solutions to allow efficient stirring. Subsequent addition of the active ester, Boc-Met-ONSu, again caused a progressive thickening of the reaction mixture. Usually a very sticky reaction mixture was obtained within 15 minutes, which could not then be stirred efficiently. It appeared that the product of the reaction, the 25-peptide III has a much greater tendency to associate in an aqueous-organic medium than the 24-peptide II. III could be isolated from the jelly-like mass after dilution with propanol-2, which induced precipitation.

The completeness of the acylation in the product III was controlled by amino acid analyses before and after dinitrophenylation. Representative results of those analyses, and similar data for the deprotected product IV, are presented in Table 2. The important figures are those for Met and Ile. The amount of methionine incorporation corresponds with the amount of isoleucine that was made unreactive towards FDNB, considering that the mean deviation of the figures is approximately 5%. The extent of acylation varied between 80 and 90% for different preparations. In further agreement, isoleucine could still be detected as an N-terminal

residue on end group determination. Complete acylation was presumably prohibited by the gelatination described above.

Table 2 AMINO ACID ANALYSES OF DERIVATIVES OF THE CYTOCHROME C SEQUENCE 81-104

Amino Acid	II	II[a]	III[1]	IV[1]	IV[1,a]	IV[1,b]	III[2]	III[2,a]	c
Asp	2.02	2.08	2.04	1.96	2.07	2.03	2.05	2.07	2
Thr	1.88	1.85	1.92	2.15	1.86	1.85	1.91	1.93	2
Glu	2.88	2.85	2.89	2.96	2.87	2.87	2.85	2.78	3
Gly	1.03	1.14	1.04	0.98	1.06	1.15	1.05	1.05	1
Ala	2.98	2.98	3.02	2.95	3.03	3.00	2.93	2.92	3
Met			0.84	0.83	0	0	0.86	0.86	
Ile	2.99	1.98	2.85	2.86	2.74	2.76	2.95	2.93	3
Leu	2.06	2.00	1.99	2.09	2.01	2.04	2.09	2.10	2
Tyr	1.00	0	1.00	0.99	0.23	0.39	1.01	0.09	1
Phe	1.01	0.98	1.03	1.00	0.98	0.98	1.02	1.05	1
Lys	4.95	4.80	4.92	4.85	4.91	0.84	4.89	4.84	5
Arg	1.03	0.97	1.03	1.03	1.06	0.96	1.02	1.04	1

1. product obtained *via* introduction of Met^{80} with Boc-Met-ONSu in aqueous DMF
2. product obtained *via* introduction of Met^{80} with Boc-Met-OPfp in DMF-DMSO

a. after dinitrophenylation
b. after removal of Msc-protective groups followed by dinitrophenylation
c. theoretical values for the sequence 81-104

Although separation of the remaining, unreacted II in the crude reaction product should be possible by ion exchange chromatography, no attempt was made to do so, since the absence of Met^{80}, one of the heme ligands, in the final 'conformational directed' coupling reaction would preclude effective complex formation.

A second route for the preparation of III was based on the observation that the 24-peptide II readily dissolved in DMSO and HMPT after pretreatment with trifluoroacetic acid. A lyophilized sample of II was dissolved at -10° in TFA, which was then removed immediately in a stream of nitrogen. Subsequent drying over moist KOH *in vacuo* gave a tough, transparant residue of II, which dissolved slowly in DMSO. It usually requir-

ed 30-60 minutes of gentle agitation for complete dissolution. In this way relatively concentrated solutions of II, up to 15% (w/v), could be obtained, which were perfectly clear and freely flowing. With these concentrated solutions the semisynthetic sequence 66-104 could be prepared in reasonable yields, even from VI and II, as noted in 5.2 (route B in Figure 1). They were also used for the condensation of II with an active ester of Boc-Met-OH.

A solution of II in DMSO, containing ca 10-15 equivalents of TFA, was neutralized with ethyldiisopropylamine. At an apparent pH of 7.5-8 (moist indicator paper) the active ester was added as a solution in DMF, and after an appropriate reaction time III, and any unreacted II, were precipitated by addition of ethyl acetate-acetic acid (9:1), in which the large amounts of ethyldiisopropylammonium trifluoroacetate, present in the reaction mixture, readily dissolve. Application of Boc-Met-ONSu as the active ester was not very successful. The coupling reaction appeared to be much more sluggish under these anhydrous conditions than in aqueous DMF; with 5 equivalents of the reagent and a reaction time of 20 hours only 45-50% of II had been acylated. Improved results were obtained using the pentafluorophenyl ester of Boc-Met-OH(Kisfaludy *et al*, 1973). Quantitative acylation was reached within 1 hour when 5 equivalents were employed. In addition to the inherent higher reactivity of this ester (Kovacs *et al*, 1980), its lypophilic character may have contributed, since the N-terminal region of the nucleophile II, H-Ile-Phe--Ala-Gly-Ile-, is lypophilic as well.

It may be noted that the reaction mixtures of the active ester coupling in DMSO-DMF also became more viscous during the course of the reaction, but not to the extent that was observed in aqueous-organic media. At present it is apparent the superior method to convert II into III.

5.4. Semisynthetic cytochrome c-(66-104)-nonatriacontapeptide (VIII)

Although the serious difficulties in the condensation between VI and II, discussed 5.2 and 5.3, could be partly overcome by performing the reaction in DMSO, yields of VII remained variable and never exceeded 37% (obtained with 70% excess of VI). The alternative route, *viz* condensations of IV and V, gave VIII in crude yields of 60-65%. In this section the performance of both, analogous condensations is described and the

resulting products are compared.

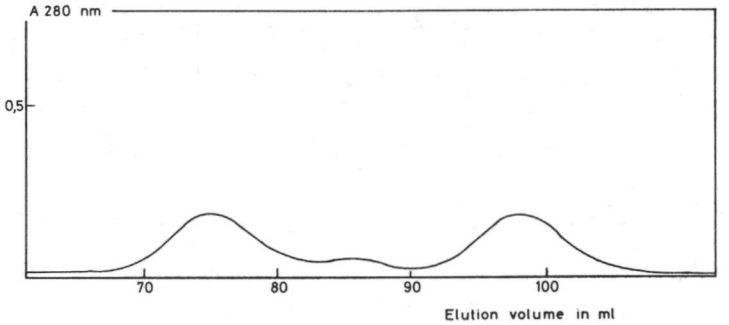

Fig 2 Isolation of VIII, prepared *via* route A, by gel filtration on Sephadex G-50 (1.4 x 110 cm) in 7% aqueous formic acid. Flow rate, 5.5 ml/h. Order of elution: 66-104, 80-104, 66-79.

The fragment condensations were performed as follows: the parent 15- and 14-peptide hydrazides were converted into the corresponding azides (VI and V) by the Rudinger procedure in DMF solution. The azides were then combined with the appropriate amino component II or III, previously dissolved in a mixture of DMSO and HMPT. The reaction mixtures were kept at 0^{o} for 2-4 days, whilst the pH was maintained at 7-8. The crude product, consisting of the newly formed 39-peptide derivative VII contaminated with the unreacted azide and amino component, was precipitated by addition of ether.

Attempts to separate the ubiquitously protected 39-peptide derivative VII by gel filtration in 50% aqueous formic acid were hampered by gelatination in this solvent. Therefore, the mixture was deprotected by treating the crude product with NaOH, added to a final concentration of 0.15-0.20N, for 30 seconds. Following neutralization with acetic acid the mixture of deprotected peptide derivatives was precipitated by addition of ether, dissolved in 7% aqueous formic acid and subjected to gel filtration on Sephadex G-50 in the same solvent system.

Figures 2 and 3 show representative elution profiles of the reaction products obtained by route A and route B, respectively. Table 3 presents amino acid analyses of the products, and of the other fractions, representing the unreacted compounds. Even without further purification the analyses agree very well with the theoretically expected values. The

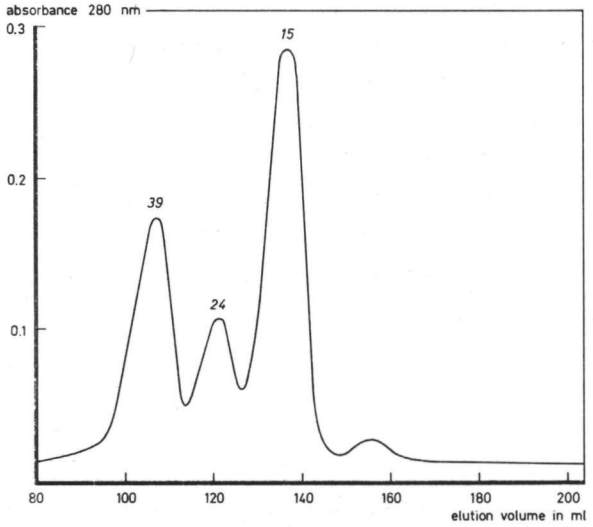

Fig 3 Isolation of VIII, prepared *via* Route B, by gel filtration on Sephadex G-50 (1.5 x 120 cm) in 7% aqueous formic acid. Flow rate, 5.8 ml/h. Order of elution: 66-104, 81-104, 66-80.

Table 3 *AMINO ACID COMPOSITION OF THE SEMISYNTHETIC CYTOCHROME C FRAGMENT 66-104 (VIII), ISOLATED BY GEL FILTRATION*

amino acid	'native' 66-104	VIII[1] 66-104	80-104[a]	66-79[a]	VIII[2] 66-104	VIII[2] 66-104	81-104[b]	81-104[b,c]	66-80[b]	66-80[b,c]
Asp	3.05	2.93	1.96	0.99	2.94	3.07	2.02	2.02	1.01	1.00
Thr	2.91	2.91	1.93	0.91	2.84	2.86	1.93	1.94	1.00	0.94
Glu	5.20	5.02	3.08	1.92	5.16	4.30	3.09	3.24	2.11	1.11
Pro	1.84	2.08		1.86	2.13	2.28			n.d.	n.d.
Gly	2.02	2.04	1.19	1.20	2.01	2.03	1.14	1.05	1.09	1.05
Ala	2.97	2.99	2.80		2.89	2.83	2.91	2.95		
Met	0.78	0.82	0.72		0.79	0.78			0.77	0.74
Ile	3.67	3.90	2.88	0.93	3.87	3.77	2.85	2.06	0.96	0.93
Leu	3.08	2.97	1.91	0.95	3.01	3.19	2.04	2.00	0.99	1.02
Tyr	2.73	2.91	1.00	1.69	2.78	0.65	0.96	0.33	1.88	0.20
Phe	0.94	0.98	0.88		0.96	0.90	0.90	0.83		
Lys	7.54	7.67	4.60	2.99	8.07	0.46	5.03	0.48	3.19	0.11
Arg	0.93	1.05	0.94		0.97	0.97	0.93	0.92		

1. obtained through route A
2. obtained through route B

a. unreacted compounds from route A (Fig 2)
b. unreacted compounds from route B (Fig 3)
c. analysis after dinitrophenylation

analyses that were obtained after dinitrophenylation of the peptides justify the conclusion that the removal of Msc-groups had been almost quantitative. This follows from the low recovery of lysine, 0.46 mole/mole of VIII, and from the decrease in recovery of glutamic acid, the N-terminal residue of VIII, by 0.86 mole per mole of VIII.

Chromatography of the semisynthetic cytochrome c sequence 66-104 on CM-cellulose (Figure 4) revealed that only very minor amounts of contaminants were present. Furthermore, the semisynthetic product was shown to co-chromatograph with the 'native' sequence 66-104.

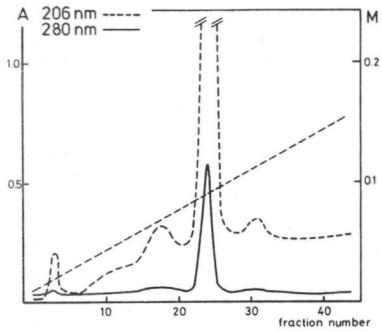

Fig 4 Purification of VIII (9 mg) on CM-cellulose (0.8 x 27 cm). Elution was with a linear sodium phosphate gradient at pH 6.9, 0.01-0.20M; flow rate: 25 ml/h; fraction volume: 4.2 ml.

5.5. Semisynthetic Hse^{65}-cytochrome c (X)

5.5.1. Preparation

The synthesis of the semisynthetic Hse^{65}-cytochrome c from the semisynthetic peptide 66-104 (VIII) and the 'native' fragment 1-65 lactone (IX) was achieved according to the optimized procedure described in Chapter II. The reaction was conducted in 0.1M sodium acetate buffer, pH 5.6, for 48 hours.

The semisynthetic protein was isolated from the reaction mixture by gel filtration on Sephadex G-50. A representative elution profile is reproduced in Figure 5. The lyophilized product was renatured by a short exposure to 8M urea at neutral pH, subjected to gel filtration on Sephadex G-25 in 0.1M sodium phosphate, pH 6.8, and subsequently chromatographed on CM cellulose in the same buffer. It is apparent from Figure 6 that the semisynthetic protein behaves identically to the native protein on ion exchange chromatography. The final yield of Hse^{65}-cytochrome c, determined spectrophotometrically, was 43%.

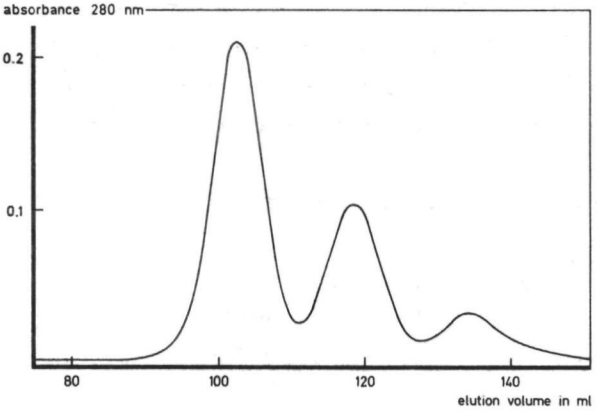

Fig 5 Isolation of Hse^{65}-cytochrome c (X) by gel filtration on Sephadex G-50 (1.3 x 120 cm) in 7% aqueous formid acid. Flow rate, 7.0 ml/h. Order of elution: 1-104, 1-65, 66-104.

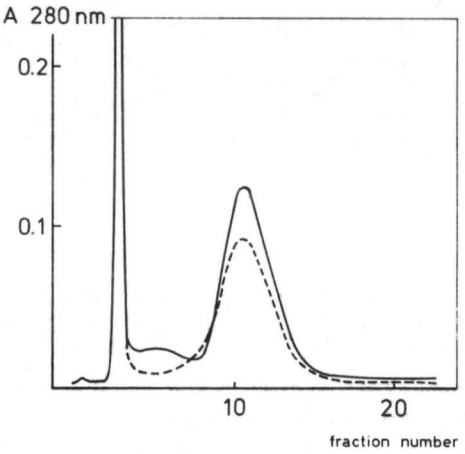

Fig 6 Elution patterns of Hse^{65}-cytochrome c, X (——), and native cytochrome c (---) on a CM-cellulose column (0.6 x 17 cm). Elution was with 0.10M phosphate buffer pH 6.9; flow rate: 10.7 ml/h; fraction volume, 2.7 ml. The profiles were matched on the potassium ferricyanide peak.

5.5.2. *Comparison with native cytochrome c*

The following properties indicate that the semisynthetic protein has a structure closely resembling that of the native protein:

1. its absorption spectrum in the ultraviolet and visible region is completely identical to that of cytochrome c, in both oxidation states. The fully developed absorption band at 695 nm is direct evidence for the coordination of the Met^{80} sulfur atom to the heme-iron
2. the semisynthetic protein is completely (>98%) reducible with ascorbate; when reduced with a large excess of ascorbate, pseudo-first order kinetics are followed throughout the time course of the reduction

and the rate constant ($31 \times 10^{-3} \cdot s^{-1}$) determined for X is equal to that ($27 \times 10^{-3} \cdot s^{-1}$) for the native protein under the same conditions (2.5 mM ascorbate, 3.0 µM cytochrome c in 0.1M phosphate pH 7.0 at 22°). These observations provide strong evidence for the homogeneity of the Hse^{65}-analogue

3. the reduced form of X does not react with carbon monoxide.

5.5.3. *Enzymatic activities of* Hse^{65}*-cytochrome* c (X)

A comparison of the functional activities of the semisynthetic Hse^{65}-cytochrome c and the native protein is given in Figure 7.

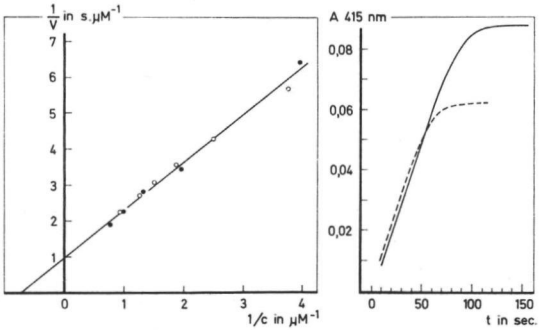

Fig 7 A: Comparison of the activity of Hse^{65}-cytochrome c, X (○), and native cytochrome c (●) towards cytochrome c oxidase. Velocity v is expressed in µmol of O_2 per sec and substrate concentration c is expressed in µM cytochrome c

B: Changes in absorbance at 415 nm when 1.8 µM ferri-Hse^{65}-cytochrome c, X (---), and 2.5 µM native ferricytochrome c (——) are reduced with a succinate cytochrome c reductase preparation.

The cytochrome c oxidase activity was determined at relatively high ionic strength in a phosphate buffer; these conditions gave rise to monophasic kinetics. A K_m-value of 1.3 µM is calculated for both proteins (Fig 7A). The value agrees well with that found by Ferguson-Miller *et al* (1976) for the second polarographic phase. The first polarographic phase, which is only apparent at low ionic strength and in non-binding buffers (Chapter VI) has also been determined; X was shown to be equally active as the native protein under these more sensitive conditions (Chapter VII, Figure 6A).

The succinate cytochrome c reductase activity was determined spec-

trophotometrically by recording the change in 415 nm or 550 nm absorbance during the enzymatic reduction. Figure 7B represents such traces for the native and semisynthetic protein. The rates at the zero order part of the reaction (*cf* Chapter II) were equal. It is clear that in the last stage of the reductions the cytochrome c concentration becomes rate limiting. In fact, *pseudo* first order kinetics are followed here, since the half logarithmic plots of the change in concentration against time eventually becomes linear (Figure 8): the rate constants calculated from that portion of the graphs were $82 \cdot 10^{-3}$ and $99 \cdot 10^{-3} \cdot s^{-1}$ for the native and semisynthetic protein, respectively. It is significant that Hse^{65}-cytochrome c was completely (>98%) reduced. Very similar results were obtained when the reduction was carried out with a NADH-cytochrome c reductase preparation (complex I + III).

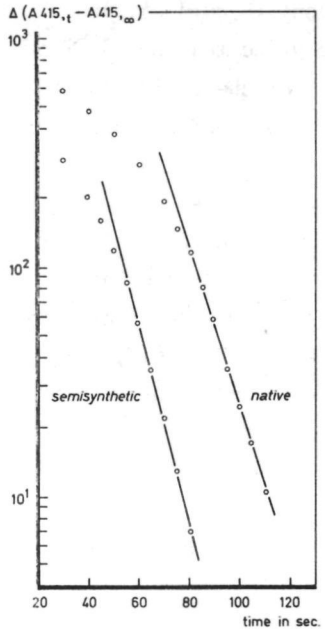

Fig 8 Semilogarithmic plots of the change in concentration of native and Hse^{65}-cytochrome c (X), measured at 415 nm (Figure 7B), on reduction with succinate cytochrome c reductase. Absorbance is in arbitrary units.

5.6. *Discussion*

The semisynthesis of Hse^{65}-cytochrome c from three fragments, described in this and preceding chapters has yielded a product that is indistinguishable from the analogue prepared from two native fragments.

Moreover, it is indistinguishable from the native protein in its ability to transfer electrons from Complex III to Complex IV in the mitochondrial electron transport chain.

This conclusion must be regarded as the most important result of the work described in this thesis, since the development of an adequate methodology for the semisynthesis of cytochrome c was the main purpose.

The Msc-protecting function played a prominent part in the strategy used. In the semisynthesis of the fragment 66-104 this group was the only protecting function that was used. This strategy of minimal side chain protection, which necessitates the use of the azide method for segment condensation, has the advantage that only one deprotection step is required to generate the desired peptide. The very brief base treatment that is sufficient to remove Msc-groups, converts the crude reaction product VII into the semisynthetic sequence 66-104 (VIII), which is almost homogeneous on ion exchange chromatography (Figure 4), and which behaves identically to the corresponding sequence that can be isolated from cytochrome c (Chapter II). The identity of the semisynthetic and native fragment was confirmed by the comparison of peptide maps of tryptic and chymotryptic hydrolysates.

The clean and smooth course of the Msc-deprotection, also observed in the deprotection of Msc-cytochrome c (Chapter III) and the synthetic peptide 66-80 (Chapter IV), favours the group above alternative base-labile amino protective functions which are in use in the semisynthesis of proteins (Sheppard, 1980), *viz* the trifluoroacetyl-, acetimidyl- and phthaloyl-group, and which are less readily removed as is apparent from the literature. For instance, the TFA-protected cytochrome c fragment 81-104 was reported to be highly resistent to basic hydrolysis (Ledden *et al*, 1979; *cf* Fanger and Harbury, 1965).

The acetimidyl function has been used frequently in recent semisynthetic studies, *e.g.* phospholipase A_2 (Slotboom and de Haas, 1975), myoglobin (Di Marchi *et al*, 1978), cytochrome c (Harris and Offord, 1977; Wallace and Offord, 1979) because it does not modify the basic character of amino functions, protected by this group; most acetimidated proteins can be studied without deprotection, since biological activity is generally retained. Removal of the acetimidyl group, although performed in the semisynthesis of myoglobin without accompanying deamidation (Di

Marchi *et al*, 1979), requires, however, exposure to concentrated ammonium hydroxide-acetic acid (15:1, v/v) at pH 11.5 for 28 hours.

A further attractive feature of the Msc-group is the ease with which it can be introduced (Chapter III). By comparison, other amino protecting groups are usually introduced by supplying enormous excesses of reagents (20-60 equivalents per lysyl residue), which consequently increase the possibility of concomittant side reactions. In a study on the acetimidation of myoglobin Di Marchi *et al* (1978) showed that under the conditions which are generally used to introduce this group, up to 5% of the lysyl residues were irriversibly modified. Since myoglobin contains 19 residues of lysine almost 50% of the crude reaction product consisted of molecules containing one or more unwanted modifications. It is noteworthy that a similar analysis of the acetimidated form of cytochrome c (Wallace and Offord, 1979) has not been reported.

This example emphasizes the importance of high specificity in reactions used for protein modifications. Msc-ONSu seems to meet these stringent requirements.

There have been several other reports in the literature concerned with semisynthetic strategies for the preparation of Hse^{65}-cytochrome c, all of which incorporate the conformationally guided coupling between 1-65 and 66-104 as the final step.

Barstow *et al* (1977) prepared the 66-104 fragment by a Merrifield solid phase procedure. They obviously used a non-optimal procedure, since Hse^{65}-cytochrome c could be prepared from it in a moderate yield of 6%. Recently it was shown by Atherton *et al* (1980) that the assembly of the nonatriacontapeptide 66-104 by the polyamide solid-phase procedure using N^{α}-fluorenylmethoxycarbonyl (Fmoc) amino acids, was identical to the natural fragment 66-104 by several analytical criteria. Although their product has not yet been reported to be coupled to 1-65, it seems to provide a promising method for the preparation of cytochrome c analogues.

In the procedure described by Nix and Warme (1979) a completely side chain protected sequence 66-79, obtained through a solid phase synthesis, was coupled as the N-hydroxysuccinimide ester to the Boc-protected sequence 80-104. The semisynthetic sequence 66-104 was liberated from the product by treatment with HBr in trifluoroacetic acid and converted

to Hse^{65}-cytochrome c in a yield of 13%. This scheme has also been used to prepare a number of cytochrome c analogues: Hse^{65},Phe^{67}-, Hse^{65},*p*-fluorophenylalanine^{67}- and Hse^{65},Leu^{74}-cytochrome c (Koul *et al*, 1979); Hse^{65},S-methyl cysteine^{80}- and Hse^{65},ethionine^{80}-cytochrome c (Wasserman *et al*, 1980). It is rather amazing that no information whatsoever is provided by these authors about the biophysical properties of the analogues, including Hse^{65}-cytochrome c. Therefore, the biological activities that have been reported for their 'purified' preparations, which were shown to be contaminated by the heme fragment 1-65 to an extent of 20-60%, do not allow relevant conclusions to be made for any of the substitutions involved.

Wallace and Offord (1979) have described methods for the semisynthesis of the sequence 66-104 of cytochrome c, and for analogues thereof, starting from the fragments 66-80 and 81-104, both of natural origin. Hse^{65}-cytochrome c, protected at the N$^{\epsilon}$-amino functions with the acetimidyl group, has been prepared in a yield of 10%.

It appears that up to now, neither of the methods reported has been shown to afford well-characterized analogues of Hse^{65}-cytochrome c. In the following chapters it will be shown that the procedures developed in Chapter II to V are well suited to that purpose.

5.7. *Experimental*

Materials

Dimethylformamide ('Baker Analyzed' reagent) was distilled and stored at 0°C over Linde 4A molecular sieve. Dimethylsulfoxide ('Merck-Schuchardt' zur Synthese) and hexamethylphosphoric triamide (HMPT, 'Merck' zur Synthese) were freshly distilled *in vacuo* before use; these solvents were stored under nitrogen in the refrigerator.

Boc-Met-OH was prepared with Boc-azide according to the 'pH-stat' method of Schnabel (1967). Boc-Met-ONSu and Boc-Met-OPfp were prepared from Boc-Met-OH according to the procedures of Anderson *et al* (1964) and Kisfaludy *et al* (1973), respectively.

N^{α}-Boc,$N^{\varepsilon 86-88,99,100}$-penta-Msc-cytochrome c-(80-104)-pentacosapeptide (III). Method 1: *via* acylation of II with Boc-Met-ONSu in aqueous DMF.

The partially protected, C-terminal cytochrome c fragment II, a lyophilisate of the DEAE-cellulose purified compound (21 mg, 5.95 µmoles), was suspended in DMF (1.0 ml). Upon addition of water (0.5 ml) a perfectly clear, but somewhat viscous solution resulted, which was further diluted with water (1.0 ml). Boc-Met-ONSu (20 mg, 58 µmoles) in DMF (0.2 ml) was added to the vigourously stirred solution. The apparent pH of the solution was kept between 8.0 and 8.5 by addition of ethyldiisopropylamine (EDPA, 10% solution in DMF). Within a few minutes the solution gelatinized. After 5 hours, 2-propanol (15 ml, peroxide free) was added to the sticky mass. After storage of the mixture at 0° for 3 hours the precipitate was collected by centrifugation, washed with 2-propanol and ether, and dried (18 mg, 80%). The extent of acylation was determined by amino acid analyses (see Table 2).

Method 2: *via* acylation with Boc-Met-OPfp in anhydrous DMF-DMSO.

The N^{ε}-Msc protected 24-peptide derivative II (100 mg, 28 µmoles, of a lyophilisate obtained from an aqueous solution) was dissolved in trifluoroacetic acid (1.0 ml) at -10°C. The solvent was subsequently evaporated in a current of nitrogen and the oily residue was dried *in vacuo* for 3 hours. The resulting tough and transparent residue (145 mg, containing at least 54 mg, ca 470 µmoles of TFA) was dissolved in a mixture of DMSO (1.30 ml) and DMF (0.50 ml). A clear solution was obtained after

gentle swirling for about 30 minutes. The solution was neutralized at 0°C by portionwise addition of a 10% solution of EDPA in DMF. When 1.03 ml of the solution of the base had been added the apparent pH (moist indicator paper) was 7.5-8.0. The amount of base added, 604 μmoles, closely corresponded with the amount calculated to be required to neutralize the TFA present (ca 470 μmoles) and the 6 acidic functions, 5 carboxylic acid side chains and the α-amino function (148 μmoles), of derivative II. Five equivalents of Boc-Met-OPfp (59 mg) in DMF (0.8 ml) were added with stirring at 0°C to the above solution. During the initial 30 minutes the apparent pH had to be restored to a value between 7.5 and 8.0 by addition of 4 portions of the 10% solution of the base. During the same time the mixture became increasingly viscous. After 5 hours at 0° a very thick, but clear, gel was obtained.

Addition of 5% acetic acid in ethyl acetate (30 ml) with stirring for 10 minutes precipitated the reaction product III. It was isolated by subsequent centrifugation and successive washings with 5% acetic acid in ethyl acetate, ethyl acetate and ether. The resulting white, fluffy precipitate was dried in a stream of nitrogen and finally *in vacuo* over moist KOH to yield III (85.3 mg). The extent of acylation was determined by amino acid analysis, presented in Table 2.

$N^{\varepsilon 86\text{-}88,99,100}$-penta-Msc-cytochrome c-(80-104)-pentacosapeptide (IV). Compound III (15-40 mg) was partially deprotected by dissolution in 90% (v/v) aqueous trifluoroacetic acid (1.0-1.5 ml) at -10°C. The solution was kept at room temperature for 40-50 minutes. The product was isolated by precipitation with ether or by removal of the trifluoroacetic acid with nitrogen. In either case, IV was dried and, before coupling to the azide V, treated with 100% trifluoroacetic acid, as described for compound II.

Cytochrome c-(66-104)-nonatriacontapeptide (VIII).

Route A: V + VI: The Msc-protected 14-peptide hydrazide V (16.8 mg, 7.0 μmoles) was dissolved in DMF (250 μl) and treated with dry HCl in ethyl acetate (14 μl of a 3.9M solution, 55 μmoles) at -20° with stirring. *tert*-Butyl nitrite (25 μl of a 4% (v/v) solution in DMF, 8.75 μmoles) was added and the solution was kept at -15°C for 15 minutes, then cooled to -30°C and neutralized by the addition of EDPA (75 μl of a 10% (v/v) solution in DMF, 43.5 μmoles).

The presence of the azide function was established by infrared spectroscopy. Part of the solution was used to acylate H-Trp-Gly-OMe in order to monitor the acylating ability of the azide.

The remainder of the activated peptide derivative V (6.62 μmoles) was added to a pre-cooled solution of IV (15 mg, 4.16 μmoles) in a mixture of DMSO (120 μl) and HMPT (100 μl). The apparent pH of the reaction mixture was adjusted to 7.0-7.5 with EDPA (92 μl of a 10% v/v solution in DMF, 53.4 μmoles). The pH had to be readjusted after ca 38 hours at 0° with the base solution. After a reaction period of 4 days the Msc-protected 39-peptide VII was precipitated by addition of 3 volumes of dry, pre-cooled ether, and dried *in vacuo*.

The crude material (30 mg) was dissolved in a mixture of DMSO (0.3 ml) and HMPT (1.2 ml). A clear solution was obtained within 30-60 minutes. The mixture was diluted with methanol (0.5 ml), whereon 4M sodium hydroxide (0.10 ml) was injected at once into the clear solution with vigorous agitation (Vortex mixing). The initial base concentration was, therefore, approximately 0.20M. After exactly 30 seconds the base was neutralized by the addition of excess acetic acid. The peptides were precipitated by the addition of pre-cooled ether and then dried. The sediment was redissolved in 7% aqueous formic acid and applied to a Sephadex G-50 column (1.3 x 125 cm) which was equilibrated and eluted with the same solvent (Figure 2). Fractions comprising the first peak were pooled. Samples were taken for amino acid analyses (Table 3); the yield of VIII was 65%, as determined from an analysis to which a known amount of norleucine had been added as an internal standard.

The semisynthetic peptide VIII was isolated by lyophilization, dissolved in water and again lyophilized. The procedure was repeated to remove excess formic acid. The fine, fluffy material (12.3 mg) was dissolved in 5.0 ml of sodium phosphate buffer (0.01M, pH 6.9) and applied to a CM-52 column (0.8 x 27 cm), equilibrated in the same buffer. The column was eluted with a gradient obtained by mixing 100 ml of the 0.01M phosphate buffer with 100 ml of the same buffer, 0.2M in sodium phosphate. Fractions comprising the main peak (Figure 4) were pooled and desalted on Sephadex G-25 (1.2 x 35 cm) with 0.1M acetic acid as the eluent. The product was isolated by lyophilization and stored at -20°C.

Route B: VI + II: A solution of the Msc-protected 15-peptide hydrazide

VI (30.0 mg, 11.7 μmoles) in DMF (250 μl) was treated with dry HCl in ethyl acetate (45 μl of a 1.3N solution, 58.5 μmoles) at -20°C with stirring.
tert Butyl nitrite (16.8 μl of a 10% solution in DMF) was added. The solution was kept at -10° - -15°C for 20 min and then neutralized with EDPA (14.1 μl, 82 μmoles).

The presence of the azide function was established by infrared spectroscopy. Part of the azide was allowed to react with an equivalent amount of H-Trp-Gly-OMe: a 30% conversion was estimated by thin layer chromatography after 20 hours.

The remainder of the solution (8.5 μmoles) was combined with a solution of the amino component II (4.8 μmoles) in DMSO (100 μl) and HMPT (50 μl), that was previously neutralized with EDPA (95 μmoles). After 40 hours at 0°C at an apparent pH of 7.0-7.5, VIII was isolated after deprotection as described for route A (Figure 3). The yield of VIII, determined by amino acid analyses, was 37%.

Semisynthetic Hse^{65}-cytochrome c (X). The cytochrome c chain was reconstituted by the procedure described in Chapter II. The 'native' (1-65)-lactone IX (7.0 mg, 0.91 μmoles) and the semisynthetic fragment 66-104 (VIII, 3.0 mg, 0.65 μmoles) were dissolved in 0.1M sodium acetate buffer pH 5.6 (2.0 ml). The mixture was reduced with the minimal amount of sodium dithionite (16 μl of a 22 mM solution, 0.35 μmoles) and kept in the reduced form for 48 hours. A 70% conversion of VIII was estimated from the absorbance change at 550 nm upon aeration. The product was isolated by gel filtration on Sephadex G-50 (Figure 5), treated with urea, and subsequently purified on CM-cellulose (Figure 6) as described for Hse^{65}-cytochrome c, prepared from the natural fragments. The yield determined spectrophotometrically was 43% (0.28 μmoles).

Cytochrome c oxidase activity. Beef heart cytochrome c oxidase was purified as described by van Buuren (1972; cf. procedure II in Hartzell *et al*, 1978). The activity was measured polarographically at 25° with a Clark electrode mounted on a Gilson oxygraph. The reaction mixtures (total volume: 1.65 ml) contained 50 nM of cytochrome c aa_3, 50 mM potassium phosphate at pH 6.8, 0.5% (v/v) Tween-20, 250 mM sucrose, 6.0 mM ascorbate and 1.0 mM N,N,N',N'-tetramethylphenylenediamine (TMPD). Cytochrome c concentrations ranged from 0.2 to 1.2 μM. The results are

presented in Figure 7A.

Succinate cytochrome c reductase activity. A succinate cytochrome c reductase preparation as described by Yu *et al* (1972) was used. The activity was measured spectrophotometrically by recording the absorbance change at 415 or 550 nm. The reaction mixtures (1.00 ml) contained 7.5 µg of the reductase, 50 mM Tris.HCl at pH 8.0, 250 mM sucrose, 10 mM succinate and 0.4 mM KCN. Cytochrome c concentrations ranged from 1.0 to 4.0 µM.

CHAPTER VI

SEMISYNTHESIS OF CYTOCHROME C ANALOGUES OF MODIFIED CHARGE AT POSITIONS 72, 73 AND 79

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FEBS Lett. *108*, 131-135.

CHAPTER VI

SEMISYNTHESIS OF CYTOCHROME C ANALOGUES OF MODIFIED CHARGE AT POSITIONS 72, 73 AND 79

6.1. Introduction

Chemical modifications have been employed frequently in studies on the relationship between the structure of cytochrome c and its physical and biological properties (see Dickerson and Timkovich, 1975 and Ferguson-Miller *et al*, 1979).

An approximate classification can be made according to the type of modification in those concerning surface residues, mainly lysines and those with more concerted residues. This latter category includes such residues as Met^{80}, Trp^{59}, the tyrosyl residues 67, 74 and 48 and the heme propionyl groups. In general, proteins modified at those positions are structurally more or less perturbed, which is apparent from such changes as: the absence of a 695 nm absorption band due to displacement of Met^{80} as the sixth heme ligand; the opening of the heme crevice; the loss of reducibility by ascorbate; carbon monoxide binding in the reduced state and autoxidizability. Since conformational changes affect the redox properties of the protein, the changes in biological properties can then not be ascribed to the pertinent local modification.

Dickerson and Timkovich (1975) have stated that only those modified proteins which exhibit the 695 nm band will be of value in elucidating the mechanism of the biological redox process. This requirement is likely to be fulfilled when modifications are limited to the surface residues of the protein.

The importance of the positively charged lysine side chains for biological activity was established when it was found that the interaction of cytochrome c oxidase (Davies *et al*, 1964; Mochan *et al*, 1973) and cytochrome c reductase (Davis *et al*, 1972) is sensitive to the ionic strength of the medium; for example, poly-lysine was shown to inhibit competitively the reaction of cytochrome c with either enzyme system.

A number of early studies was devoted therefore to the effects of modification of the lysine side chains in cytochrome c, *e.g.* following

succinylation, acetylation or trinitrophenylation (Dickerson and Timkovich, 1975). Those studies indicated that removal of the charge from 4 to 6 of the lysyl residues made the protein unreactive towards the oxidase. Only Lys^{13} was firmly identified as belonging to those residues; this was demonstrated by Margoliash *et al* (1973) who prepared the 4-nitrobenzo-2-oxa-1,3-diazole (NBD) Lys^{13} derivative. On the contrary, the modification of Lys^{13} did not seem to interfere with the interaction of cytochrome c with a succinate cytochrome c reductase preparation. Investigations with differently modified cytochromes c (*i.e.* Aviram and Schejter, 1973), antibody-binding studies (Smith *et al*, 1973a) and kinetic studies (Smith *et al*, 1974) also pointed to the existence of two different binding sites on cytochrome c for the oxidase and reductase.

In the strategy that was developed for the semisynthesis of Hse^{65}-cytochrome c, described in the preceding chapters, the opportunities for the preparation of well defined analogues of the protein concern primarily the synthetic sequence 66-80. Since the critical step in this strategy is dependent on the correct positioning of the fragments 1-65 and 66-104 in the final recombination step, it was decided to prepare a number of analogues of the protein with charge modifications at positions 72, 73 and 79. It was anticipated that such surface modifications would not interfere in the final step. Furthermore, several studies (Aviram and Schejter, 1973; van Gelder *et al*, 1976) had already suggested a possible involvement of these residues in the interaction of cytochrome c with its biological redox partners.

The acetyl group was chosen for the charge modifications since it would retain part of the polar character of the lysyl side chains and it was expected not to interfere sterically on account of its small size.

In this chapter the semisynthesis of seven acetylated analogues of Hse^{65}-cytochrome c will be described, followed by a comparison of their activities towards cytochrome c oxidase. The effects of the modifications of Lys^{72}, Lys^{73} and Lys^{79} will be compared with the results of a number of other recent studies with singly modified cytochromes c.

6.2. Synthesis of seven selectively acetylated analogues of the cytochrome c-(66-79)-tetradecapeptide derivative 38

The strategy described for the synthesis of the tetradecapeptide 38 (Chapter IV, Scheme 1) has been employed to prepare the seven analogues 38a-g (Figure 1).

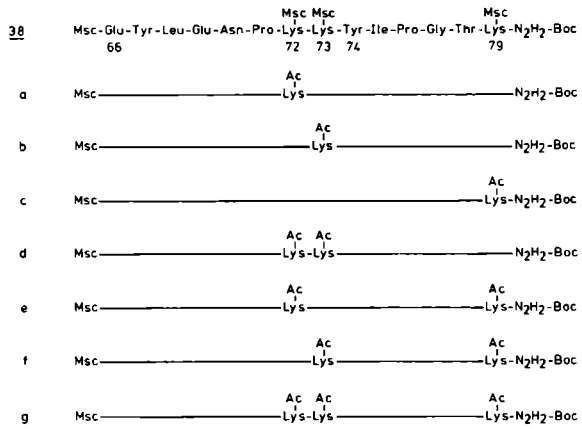


Fig 1 Seven tetradecapeptide derivatives 38a-g for covalent insertion between the isolated cytochrome c fragments 1-65 and 80-104.

The desired variants of 38 represent replacements of one or more of the N^{ϵ}-Msc-lysyl residues by N^{ϵ}-acetyl-lysyl residues. The variants were obtained by the preparation of the tripeptide Z-Gly-Thr-Lys(Ac)-N_2H_2-Boc, 44, already described in Chapter IV (Scheme 6), and of three acetyl containing analogues of the cytochrome c-(72-76)-pentapeptide derivative 24. The title compounds were then obtained by the condensation of the appropriate subfragments *via* two successive azide coupling reactions.

6.2.1. Cytochrome c-(72-76)-pentapeptide derivatives

Several N^{ϵ}-acetyl and N^{ϵ}-t-butyloxycarbonyl derivatives of lysine were prepared (Figure 2). The acetyl containing derivatives 47, 48 and 49, which have not been described previously, were obtained in good yields, and analytically pure, using established procedures (see 6.5: Experimental). The lysine derivatives 47, 48, 51 and 52 were used to prepare the cyrstalline dipeptide hydrazides 54, 56 and 58 as shown in Figure 3.

The fully protected pentapeptide *t*-butyl esters 61, 62 and 63 (Figure 4) were prepared by two methods:

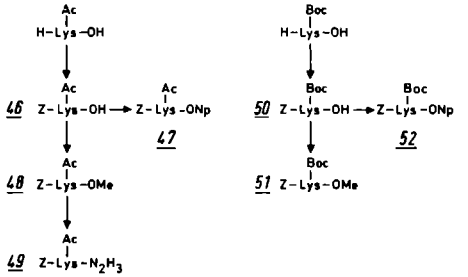


Figure 2

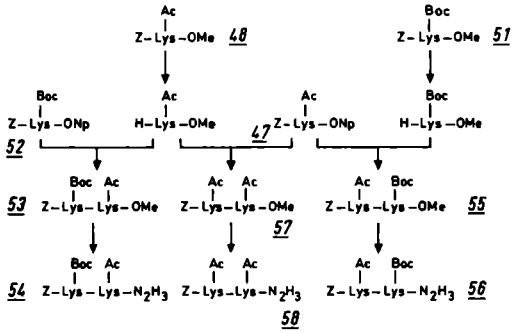


Figure 3

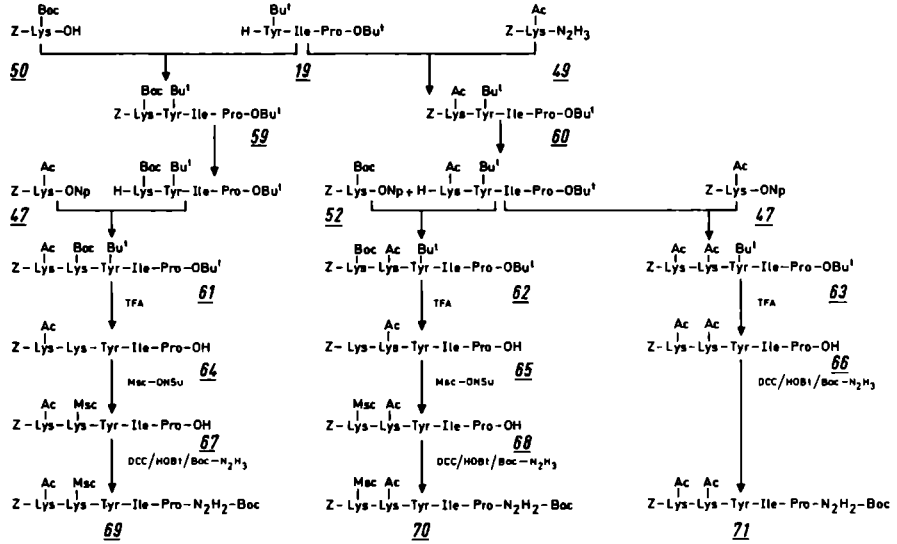


Figure 4

Table 1 ANALYTICAL DATA OF INTERMEDIATES (Figure 3 and 4)

	Compound	Formula	Elemental analysis	C	H	N	m.p.
53	Boc Ac Z-Lys-Lys-OMe	$C_{28}H_{44}O_8N_4$	Calc	59.56	7.85	9.92	125-128°
		564.680	Found	59.21	7.82	9.75	
54	Boc Ac Z-Lys-Lys-N_2H_3	$C_{27}H_{44}N_6O_7 \cdot \frac{1}{2}H_2O$	Calc	56.53	7.91	14.65	146-148°
		573.693	Found	56.74	7.86	14.49	
55	Ac Boc Z-Lys-Lys-OMe	$C_{28}H_{44}N_4O_8 \cdot \frac{1}{2}H_2O$	Calc	58.62	7.91	9.77	120-121°
		573.688	Found	58.64	7.88	9.74	
56	Ac Boc Z-Lys-Lys-N_2H_3	$C_{27}H_{44}N_6O_7 \cdot 1H_2O$	Calc	55.65	7.96	14.42	187-189°
		582.701	Found	55.81	7.73	14.47	
57	Ac Ac Z-Lys-Lys-OMe						174-176°
58	Ac Ac Z-Lys-Lys-N_2H_3	$C_{24}H_{38}N_6O_6 \cdot 1H_2O$	Calc	54.95	7.68	16.02	227-230°
		524.620	Found	54.97	7.43	15.89	
59	Boc Bu^t Z-Lys-Tyr-Ile-Pro-OBu^t	$C_{47}H_{71}N_5O_{10}$	Calc	65.18	8.26	8.09	138-141°
		866.110	Found	65.29	8.29	8.08	
60	Ac Bu^t Z-Lys-Tyr-Ile-Pro-OBu^t	$C_{44}H_{65}N_5O_7$	Calc	65.40	8.11	8.67	165-166°
		808.030	Found	64.77	7.71	8.47	
61	Ac Boc Bu^t Z-Lys-Lys-Tyr-Ile-Pro-OBu^t	$C_{55}H_{85}N_7O_{12}$	Calc	63.75	8.27	9.46	186-189°
		1036.322	Found	63.83	8.36	9.58	
	Ac Boc Bu^t Z-Lys-Lys-Tyr-Ile-Pro-OBu^t	$C_{55}H_{85}N_7O_{12}$	Calc	63.75	8.27	9.46	183-185°
		1036.322	Found	63.25	8.36	9.30	
62	Boc Ac Bu^t Z-Lys-Lys-Tyr-Ile-Pro-OBu^t	$C_{55}H_{85}N_7O_{12}$	Calc	63.75	8.27	9.46	186-187.5°
		1036.322	Found	63.82	8.38	9.46	
63	Ac Ac Bu^t Z-Lys-Lys-Tyr-Ile-Pro-OBu^t	$C_{52}H_{79}N_7O_{11}$	Calc	63.85	8.14	10.02	191-193°
		978.242	Found	63.54	8.24	9.99	
66	Ac Ac Z-Lys-Lys-Tyr-Ile-Pro-OH						90-120°
67	Ac Msc Z-Lys-Lys-Tyr-Ile-Pro-OH	$C_{46}H_{67}N_7O_{14}S \cdot 1H_2O$	Calc	55.68	7.01	9.88	92-115°
		992.157	Found	55.55	6.85	9.76	
68	Msc Ac Z-Lys-Lys-Tyr-Ile-Pro-OH						95-115°
69	Ac^t Msc Z-Lys-Lys-Tyr-Ile-Pro-N_2H_2-Boc	$C_{51}H_{77}N_9O_{15}S \cdot 1H_2O$	Calc	55.37	7.20	11.40	>111° dec
		1106.305	Found	55.18	7.15	10.89	
70	Msc Ac Z-Lys-Lys-Tyr-Ile-Pro-N_2H_2-Boc	$C_{51}H_{77}N_9O_{15}S \cdot 1H_2O$	Calc	55.37	7.20	11.40	>122° dec
		1106.305	Found	55.14	7.22	11.71	
71	Ac Ac Z-Lys-Lys-Tyr-Ile-Pro-N_2H_2-Boc	$C_{49}H_{73}N_9O_{12} \cdot 1H_2O$	Calc	58.96	7.57	12.63	>215° dec
		998.190	Found	58.87	7.61	12.61	

Table 1 (continued)

$[\alpha]_D^{22^\circ}$	TLC[1]			Yield	Remarks[2]
	A	B	C		
-12.8° (c=0 98, MeOH)	0.69	0.77	0.76	74%	triturated with ether
-19.7° (c=0.95; MeOH)		0.40	0.52		recrystallized from methanol/water
-12.3° (c=0.95, MeOH)	0.70	0.74	0.73	89%	triturated with ether
-19.6° (c=1.15, MeOH)		0.43	0.57		recrystallized from methanol/water
-13.8° (c=0.87, MeOH)		0.58	0.57	80%	triturated with ether, recrystallized from propanol-2-water
-16.4° (c=1.10, MeOH)		0.15	0.35	95%	crystallized from methanol
-67.0° (c=1.00, MeOH)	0.84		0.90	74%	chromatography on silica ($CHCl_3$/methanol=99/5), precipitated from ethyl acetate/hexane
-64.8° (c=1.00, MeOH)	0.75	0.79	0.81	69%	crystallized from ethyl acetate
-56.6° (c=0.91, MeOH)	0.77		0.87	72%	crystallized from methanol/diisopropylether, prepared from 47 and 59
-57.2° (c=1.17; MeOH)		0.67	0.86	69%	crystallized from methanol, prepared from 56 and 19
-57.4° (c=0.93; MeOH)	0.78		0.84	76%	triturated with ether, crystallized from methanol/diisopropylether
-61.3° (c=0.45; MeOH)	0 74	0.71	0.72	85%	triturated with ether
-58.2° (c=0.50, MeOH)			0.60	97%	precipitated from methanol solution with ether
-51.1° (c=0.88, MeOH)			0.62	88%	precipitated from methanol solution with ether
-48.5° (c=0.92, MeOH)			0.64	90%	precipitated from methanol solution with ether
-70.7° (c=0.94, MeOH)	0.46		0.71	50%	chromatography on silica ($CHCl_3$/methanol=93/7), precipitated from methanol solution with ether
-74.9° (c=1.12, MeOH)	0.47		0.71	66%	chromatography on silica ($CHCl_3$/methanol=93/7), precipitated from methanol solution with ether
-53.3° (c=1.07, DMF)	0.44		0.69	43%	chromatography on silica ($CHCl_3$/methanol=90/10), slightly soluble in methanol

[1] Solvent systems A, B and C are specified in Appendix.
[2] For general procedures, see Chapter IV, 4.4.

1. *via* acylation of the tripeptide ester 19 (cf Chapter IV, Scheme 3) with azides prepared from the hydrazides 54, 56 and 58
2. *via* stepwise elongation of tripeptide 19 with the appropriate lysine derivatives.

The products obtained *via* either route were similar with regard to their physical properties. The stepwise synthesis was to be preferred, however, since the products obtained *via* the azide coupling usually contained a minor contaminant that could only be removed satisfactorily by column chromatography on silica gel. The peptides obtained *via* acylation with the p-nitrophenyl ester 47 and 52 were analytically pure after the normal work-up procedure in 1-butanol, followed by trituration with ether. Stepwise syntheses of 61, 62 and 63 using the p-nitrophenyl esters 47 and 52 also in the preparation of the intermediate tetrapeptides 59 and 60, would presumably be more simple.

The *t*-butyl and Boc-functions of 61, 62 and 63 were removed by treatment with 90% aqueous trifluoroacetic acid. The ensuing N^{α}-Z,N^{ε}-acetyl pentapeptide derivatives 64, 65 and 66 were isolated by ether precipitation in yields of 90-95%. The introduction of the Msc-group into the ε-amino functions in 64 and 65 was done as described for Z-Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro-OH (23, Chapter IV, Scheme 3), except for the use of 1-butanol, instead of ethyl acetate, in the isolation procedure.

The pentapeptide derivatives 66, 67 and 68 were subsequently converted into the corresponding *t*-Boc protected hydrazides with the aid of DCC and HOBt. The acetylated cytochrome c-(72-76)-pentapeptide derivatives 69, 70 and 71 were purified by chromatography on silica gel. The peptide derivative 71, which contains two acetyl groups, had a much lower solubility and a much higher melting point than the analogous peptides 69, 70 and 24.

The actual synthetic procedures that were followed in the preparation of the compounds shown in Figures 3 and 4 will not be described in detail here. Relevant analytical data and physical properties of the isolated intermediates are given in Table 1.

6.2.2. Cytochrome c-(66-76)-undecapeptide derivatives

X Y

Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-N_2H_2-Boc

35a: X=Ac, Y=Msc; 35b: X=Msc, Y=Ac; 35c: X=Y=Ac.

Compounds 69, 70 and 71 were deprotected at the α-amino function by catalytic hydrogenolysis in methanol-acetic acid (2:1) for 1 hour and subsequently acylated with the azide prepared from Msc-Glu-Tyr-Leu-Glu--Asn-Pro-N_2H_3 (14a, Scheme 2 in Chapter IV)

The use of a 10% excess of the azide usually resulted in complete conversion of the amino component within 16 hours at an apparent pH of 6 in DMF solution. Since the crude reaction products were relatively pure and the condensations were performed on a moderate scale (100-200 mg), chromatographically homogeneous undecapeptides could be obtained directly by gel filtration on Sephadex LH-20 using pure DMF as the solvent. The products were isolated from methanol solutions by precipitation with ether. The physical properties of the three acetylated undecapeptide derivatives 35a-c are compared with those of the completely Msc-protected analogue 35 in Table 2.

Table 2 ANALYTICAL DATA OF THE INTERMEDIATES 35a-c

	35	Ac^{72} 35a	Ac^{73} 35b	$Ac^{72,73}$ 35c
Amino acid composition				
Asp (1)	0.98	1.05	1.02	1.04
Glu (2)	1.96	2.02	2.02	2.13
Pro (2)	2.11	1.88	1.88	2.03
Ile (1)	0.95	0.98	0.97	0.94
Leu (1)	1.00	1.03	1.02	1.01
Tyr (2)	1.96	1.99	1.98	1.96
Lys (2)	2.03	1.93	1.99	1.92
NH_3 (1)	n.d.	1.06	1.04	1.08
$\|\alpha\|_D^{22}$ c=1.0; MeOH	-67.5°	-70.1°	-72.4°	-75.9°
purification	CCD[a]	LH-20 in DMF	CCD[a]	LH-20 in DMF
yield	73%	91%	77%	87%

TLC: Rf=0.40 (C); =0.57 (H) for all the compounds; the corresponding free hydrazides moved with Rf=0.25 (C) and Rf=0.43 (H)

a: CCD=counter current distribution in the solvent system butanol-1-ethyl acetate-methanol-acetic acid-water (2:2:0.5:1:7.5).

Table 3 ANALYTICAL DATA OF THE CYTOCHROME C-(66-79)-TETRADECAPEPTIDE DERIVATIVES 38a-g

		38	Ac^{72} 38a		Ac^{73} 38b		Ac^{79} 38c		$Ac^{72,73,79}$ 38g		$Ac^{72,73}$ 38d	$Ac^{72,79}$ 38e	$Ac^{73,79}$ 38f
			[1]		[1]		[1]		[1]				
Asp	(1)	1.03	1.02	1.01	1.03	0.98	1.04	1.06	1.03	1.02	1.08	1.08	1.08
Thr	(1)	0.90	0.78	0.73	0.86	0.93	0.80	0.94	0.97	0.94	0.82	0.86	0.89
Glu	(2)	2.15	2.01	1.07	1.90	1.10	1.95	1.03	2.01	1.05	2.10	2.15	2.13
Pro	(2)	1.98	1.83	2.18	2.28	n.d.	n.d.	n.d.	1.81	2.09	1.99	2.21	2.05
Gly	(1)	1.05	0.82	0.82	0.97	0.97	0.87	0.98	1.01	0.98	0.93	0.94	0.91
Ile	(1)	0.96	0.97	0.98	1.04	0.95	1.01	0.96	0.96	0.96	1.02	0.98	1.01
Leu	(1)	0.99	1.01	1.01	1.06	0.99	1.05	1.01	1.00	1.02	1.10	1.08	1.07
Tyr	(2)	1.96	1.87	0	1.97	0	1.99	0.05	1.84	0	1.96	1.99	1.95
Lys	(3)	2.87	2.70	1.02	2.89	1.01	2.82	0.90	2.81	2.75	2.99	2.92	2.96
$\lvert\alpha\rvert_D^{22}$ c=±1.0 MeOH	[2]	n.d. -32.6°	-51.4° -36.4°		n.d.° -31.6°		n.d. -30.1°		-53.3° -37.7°		-51.4° -36.4°	-51.2° n.d.	-52.0° -37.2°
yield:	[3]	75%	82%		±80%		88%		85%		±80%	±80%	±80%
	[4]	60%			53%		59%						

TLC: Rf=0.34 (C) and Rf=0.54-0.56 (H) for all the compounds; the corresponding free hydrazides moved with Rf=0.11 (C) and Rf=0.36-0.38 (H)

[1] analysis after removal of Msc-groups, followed by dinitrophenylation
[2] optical rotation of the free hydrazides, liberated from the parent peptides 38a-g with TFA
[3] yields are based on the amounts of 35a-c used in the azide coupling reactions
[4] yields after counter current distribution of the free hydrazides corresponding to 38, 38b and 38c in the solvent system butanol-1-acetic acid-water (8:1:10)

6.2.3. Cytochrome c-(66-79)-tetradecapeptide derivatives 38a-g

Each of the four undecapeptide derivatives 35, 35a, 35b and 35c was deprotected in 90% aqueous trifluoroacetic acid, converted into the corresponding azide and coupled with the amino components H-Gly-Thr-Lys(Msc)-N_2H_2-Boc (32a, derived from 32 or 43) and H-Gly-Thr-Lys(Ac)-N_2H_2-Boc (obtained by deprotection of 44). A 10-30% excess of the amino components was used in order to complete the conversion of the acylating azide (cf Chapter IV, 4.3.5). The reaction mixtures were stored up at 0° for 20 hours and then transferred to a Sephadex LH-20 column, which was eluted with pure DMF. The resulting products were usually almost homogeneous. When TLC of the corresponding free hydrazides, obtained by treatment of the products with 90% trifluoroacetic acid, revealed that more than a trace of an impurity resulting from the parent undecapeptide was present, the compounds were purified further by counter current distribution in butanol-1-acetic acid-water (8:1:10). In this way 38b and 38c were obtained as completely homogeneous compounds, but having a free hydrazide function.

Amino acid analyses of 38 and 38a-g, together with other relevant data, are given in Table 3. The analyses agree well with the expected ratios. The amino acid analyses of 38a-g, after deprotection of Msc-groups and dinitrophenylation, show that the Msc groups could be removed selectively, without affecting the N^{ϵ}-acetyl groups.

6.2.4. Semisynthetic cytochrome c-(66-104)-nonatriacontapeptide analogues

The condensations of the synthetic peptide derivatives 38a-g with the N^{ϵ}-Msc-protected cytochrome c-(80-104)-pentacosapeptide IV were performed essentially as described for the preparation of VIII in Chapter V. The syntheses were performed with 4.5-5.0 μmoles of IV (concentration approximately 10 mM) and a 50% excess of the acylating azide.

The crude reaction products were deprotected by a brief (30 seconds) treatment with base (0.15-0.20N) and then separated by gel filtration on Sephadex G-50 in 7% aqueous formic acid. The elution profiles for the three mono-acylated 39-peptides and for the analogue containing three acetyl groups are reproduced in Figure 5.

Appropriate fractions were pooled. A sample was withdrawn, supplied with a known amount of norleucine, and subjected to amino acid analysis.

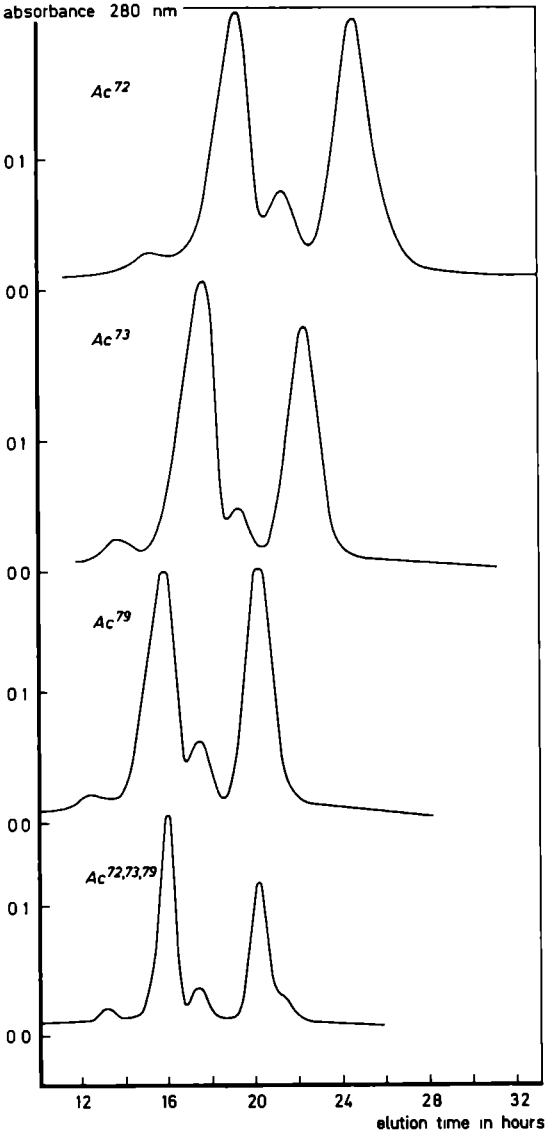


Fig 5 Isolation of semisynthetic cytochrome c-(66-104)-nonatriacontapeptide analogues by gel filtration on Sephadex G-50 (2.0 x 120 cm) in 7% aqueous formic acid. Flow rates: Ac^{72}, 5.6 ml/h; Ac^{73}, 6.1 ml/h, Ac^{79}, 6.9 ml/h; $Ac^{72,73,79}$, 6.9 ml/h.

Table 4 gives the results for each of the 7 analogues prepared. The yields of the Ac^{72}, Ac^{73}, Ac^{79} and $Ac^{72,73,79}$ derivatives, based on the recovery of the internal standard norleucine, were 48%, 55%, 48% and 64%, respectively. Amino acid analyses after dinitrophenylation of the analogues are also given in order to demonstrate that deprotection of the amino function of Glu^{66} and of the N^{ϵ}-Msc-lysyl residues was complete. For comparison an analysis of the native fragment 66-104 is also presented in Table 4. Amino acid ratios determined for each analogue agree closely with the theoretical values.

The high molecular weight compound, detected in all the crude products (Figure 5), but accounting for only a few percent of the yield, must be regarded as a by-product of the condensations. Amino acid analyses of this fraction (Table 4) indicate a product consisting of two 25-peptides and one 14-peptide. The formation of such a by-product can be envisaged when anhydride formation between a carboxyl group of the 14-peptide azide and some

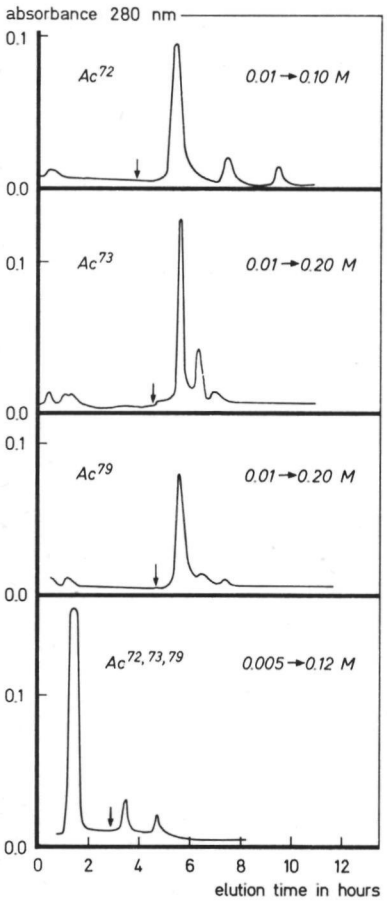
Table 4 AMINO ACID COMPOSITIONS OF CYTOCHROME C-(66-104)-NONATRIACONTAPEPTIDE ANALOGUES

Amino acid	native	Ac^{72}		Ac^{73}		Ac^{79}		$Ac^{72,73,79}$		$Ac^{72,73}$	$Ac^{72,79}$	$Ac^{73,79}$	by-product [c]		
	[a]		[b]		[b,d]		[b]		[b]		[b]			b,d	
Asp	3	3.05	3.08	3.12	3.02	2.98	3.11	2.97	2.95	2.99	3.16	3.07	3.09	4.99	5.07	5
Thr	3	2.91	2.87	2.93	2.92	2.85	2.94	2.82	2.85	2.84	2.75	2.86	2.61	5.05	4.80	5
Glu	5	5.20	4.95	4.19	4.81	4.08	5.04	4.11	4.89	3.95	5.23	4.20	4.81	7.82	7.07	8
Pro	2	1.84	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.94	n.d.	n.d.	1.91	2.11	2
Gly	2	2.02	2.04	2.07	2.09	2.09	2.01	2.10	2.13	2.09	2.01	2.07	2.13	3.32	3.31	3
Ala	3	2.97	3.00	2.99	2.89	2.92	2.86	2.96	3.03	3.03	2.93	2.95	3.02	5.77	5.84	6
Met	1	0.78	0.81	0.86	0.79	0.76	0.85	0.83	0.82	0.89	n.d.	0.96	0.75	1.18	1.21	2
Ile	4	3.67	3.82	3.70	3.80	3.78	3.78	3.81	3.82	3.74	3.71	3.64	3.41	6.61	6.62	7
Leu	3	3.08	2.96	2.85	3.01	3.01	3.04	2.99	2.98	2.95	3.07	3.05	2.97	4.99	4.86	5
Tyr	3	2.73	2.83	0.19	2.80	0.80	2.79	0.31	2.82	0.15	2.89	0.22	2.86	3.81	0.81	4
Phe	1	0.94	0.95	0.94	0.98	0.97	1.00	1.01	0.94	0.96	0.82	0.84	0.81	1.98	1.94	2
Lys	8	7.54	7.80	1.18	7.45	1.41	7.58	1.08	7.70	3.09	7.66	2.09	7.42	12.27	1.66	13
Arg	1	0.93	0.96	1.03	1.02	1.01	0.98	0.98	0.94	0.97	1.04	1.00	0.97	1.95	1.96	2

a: theoretical figures for the cytochrome c sequence 66-104
b: analysis after dinitrophenylation
c: amino acid composition of the high molecular weight by-product, isolated from the crude reaction products of the Ac^{73}-analogue (Figure 5). The theoretical figures are those for a peptide consisting of the sequence 66-79 and two sequences 80-104
d: results after relatively short treatment with FDNB, resulting in too high recoveries of Tyr and Lys

leaving group, possibly the azide itself, constitutes a side reaction during the azide coupling reaction.

The four analogues mentioned above were purified by ion-exchange chromatography on CM-cellulose. The elution profiles are reproduced in Figure 6. In each case two, more positive, by-products could be removed. Amino acid analyses of the major by-product did not differ significantly from that of the desired product. No further attempts were made to characterize the by-products.



Peptide maps of tryptic digests (Ferguson-Miller *et al*, 1978) of the mono-acetylated 39-peptides were made. The two analogues containing the acetyl group on Lys^{72} and Lys^{73} gave a pattern that could not be distinguished from that of the native fragment 66-104. Although in both cases the tryptic digest should contain one peptide having an additional N^{ε}-acetyl residue in comparison with the digest of the native 66-104, the pertinent peptides apparently did not differ in chromatographic mobility. As expected, the map of the Ac^{79}-analogue was clearly different because the peptide corresponding to the sequence 80-86 was absent in the digest.

Fig 6 Purification of semisynthetic cytochrome c-(66-104)-nonatriacontapeptide analogues by chromatography on CM-cellulose (1.0 x 1.3 cm). Elution with sodium phosphate buffers pH 6.9: a linear gradient in phosphate, obtained by mixing two solutions of indicated molarity (100 ml each), was applied at the arrows. Flow rates: 20 ml/h.

6.3. *Semisynthetic Hse^{65}-cytochrome c analogues with charge deletions at positions 72, 73 and 79*

6.3.1. *Preparation*

The conformationally directed condensations of the natural sequence (1-65)-lacone with each of the seven available acetylated fragments 66-104, the subsequent isolation of the products by gel filtration and their purification on CM-cellulose were performed as described previously (Chapter V).

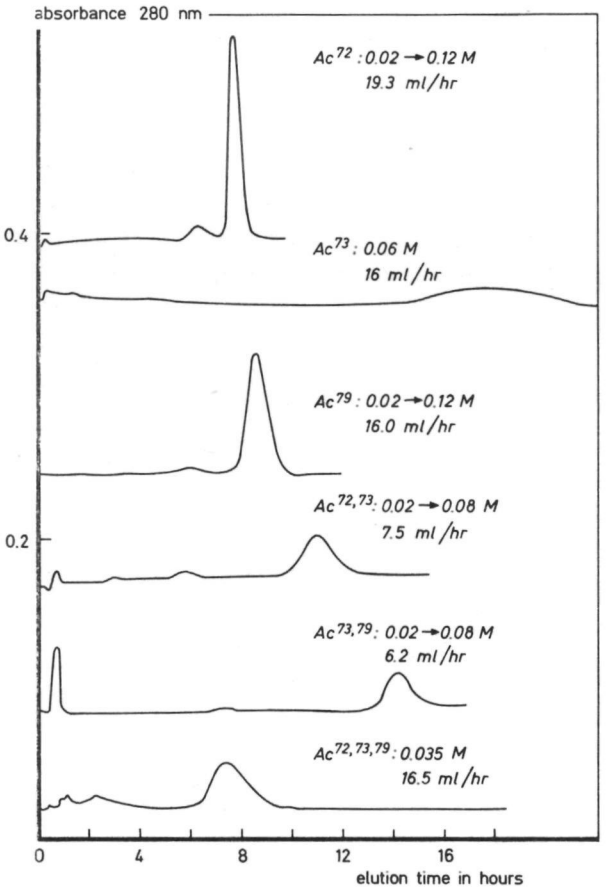


Fig 7 Purification of cytochrome c analogues on CM-cellulose (0.8 x 2.6 cm). Elution was with sodium phosphate buffer, pH 6.9, at constant ionic strength or by application of a linear gradient in phosphate, as indicated next to the elution profiles.

The presence of the acetyl groups did not influence the ability of the 39-peptides to couple with 1-65: the efficiency of the condensations proved to be comparable with that obtained with the native fragment 66-104, when the same batch of the (1-65)-lactone was used.

The elution profiles of the final purifications on CM-cellulose of six of the acetylated cytochrome c analogues are reproduced in Fig 7. The 72,79-bis N^{ε}-acetyllysine analogue was lost inadvertedly at this stage; the synthesis has not so far been repeated. The varying elution conditions are indicated in Fig 7. The profiles demonstrate that all analogues were ultimately obtained as apparently

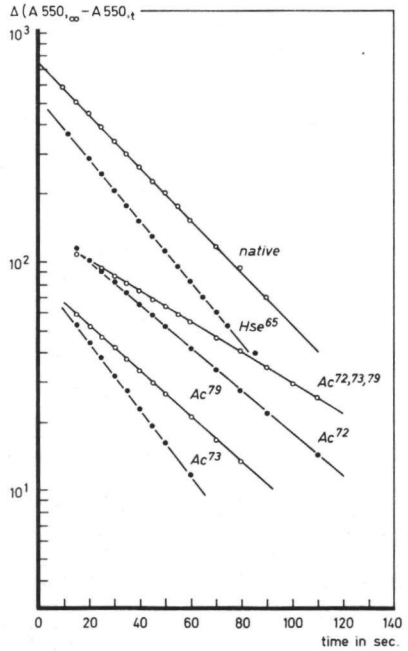
homogeneous products.

The yields of the purified analogues, ranging between 21 and 33% are comparable with that of the preparation of Hse^{65}-cytochrome c (28%, using the same batch of the (1-65)-lactone).

6.3.2. Properties of acetylated cytochromes c

Each of the six acetylated cytochromes c has the same visible spectrum as the native protein. The conformationally sensitive 695 nm absorption band is always present with the normal intensity, indicating that Met^{80} is ligated to the heme iron.

All the analogues are completely (95-98%) and rapidly reducible with ascorbate. Figure 8 shows that the reduction of the mono-acetylated and the tri-acetylated analogues with a large excess of ascorbate follows *pseudo* first order kinetics throughout the time course. The following *pseudo* first order rate constants were calculated from the graphs:



Cytochrome c	k' x 10^3
native	27 s^{-1}
Hse^{65}-	31 s^{-1}
Hse^{65},Ac^{72}-	22 s^{-1}
Hse^{65},Ac^{73}-	34 s^{-1}
Hse^{65},Ac^{79}-	23 s^{-1}
Hse^{65},$Ac^{72,73,79}$-	16 s^{-1}

Fig 6 Semilogarithmic plots of the change in concentration of ferricytochromes c, measured at 550 nm, on reduction with ascorbate (2.5 mM) at 22° in 0.1M sodium phospate buffer, pH 6.8. Concentration of cytochromes c: 2.2-4.2 μM.

This behaviour constitutes firm evidence that the analogues are homogeneous. The reduced forms did not bind carbon monoxide.

The properties also indicate that the spatial arrangement of native cytochrome c is retained in all the acetylated derivatives. It may therefore be concluded that changes in the electron transfer acitivities of the acetylated analogues when present may be ascribed to the localized charge modifications at the surface of the protein.

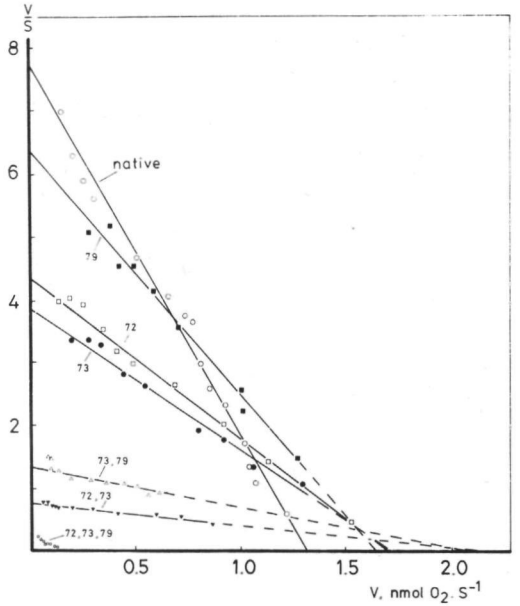
6.3.3. *Effects of acetylation of lysines 72, 73 and 79 on the rate of reaction of cytochrome c with cytochrome c oxidase*

The reaction between reduced, acetylated cytochromes c and cytochrome c oxidase was assayed polarographically, using the method of Ferguson-Miller *et al* (1976). They have observed that under conditions of low ionic strength, in non-binding buffers and at low concentrations of cytochrome c, the reaction shows biphasic kinetics. Direct binding studies have indicated that the K_m-values that characterize the two kinetic phases correlate closely with the dissociation constants of a high and a low affinity site for cytochrome c on the oxidase. The high affinity phase of the reaction appeared to be very sensitive to changes in ionic strength of the medium and to the charge distribution on cytochrome c (Ferguson-Miller *et al*, 1976, 1978).

The results for the six acetylated cytochrome analogues, presented as Eadie-Hofstee plots, are given in Figure 9. At the very low (0.01-1.0 μM) concentrations used, only the high affinity phase of the reaction is studied.

Extrapolation of the monophasic plots of the mono-acetylated proteins results in V_{max}-values which are nearly equal, but which are ca 25% higher than that for the native protein. The influence of mono-acetylation on the apparent K_m-values for this phase varied, however. Acetylation of Lys^{79} in cytochrome c has a definite, but moderate effect on the binding affinity: the K_m-value increases by ca 50%.

Acetylation of either Lys^{72} or Lys^{73} elicits a larger influence: K_m-values are 2-2.5 fold in comparison with native cytochrome c. The difference between the apparent importance of the charges at Lys^{72} or Lys^{73} and Lys^{79} is also reflected in the relative activities of two di-acetyl derivatives: the K_m-value for the $Ac^{73,79}$-analogue is 8 times higher, that of the $Ac^{72,73}$-analogue ca 16 times higher than the K_m-value determined for the native protein. It is striking that the elimination of



derivative	K_m *in* µM
native	0.17
Hse^{65}	0.17
Hse^{65},Ac^{79}	0.26
Hse^{65},Ac^{72}	0.39
Hse^{65},Ac^{73}	0.44
Hse^{65},$Ac^{73,79}$	1.4
Hse^{65},$Ac^{72,73}$	2.8
Hse^{65},$Ac^{72,73,79}$	>>

Fig 9 Steady-state cytochrome oxidase activities of acetylated cytochrome c derivatives at low cytochrome c concentrations (0.01-1.0 µM). The sites of acetylation are marked next to the plots. Velocities are in nmoles O_2 reduced per second and S is the concentration of cytochrome c in micromoles·l^{-1}. The assay medium, 25 mM Tris-acetate, pH 7.8 (1.25 ml), contained in addition to cytochrome c: 250 mM sucrose, 0.1% Tween-20, 20 mM ascorbate, 2 mM TMPD and 0.114 µM of a purified cytochrome c oxidase preparation.

of a second charge affects the binding, in both analogues studied, to a much larger degree than the first charge deletion. Removal of a third charge gives an analogue which hardly binds to the oxidase, as is apparent from the very low activity of the $Ac^{72,73,79}$-analogue.

6.4. Discussion

The strategy for the semisynthesis of cytochrome c from three fragments can readily be applied to produce well-defined analogues of Hse^{65}-cytochrome c, which are inaccessible or not readily obtainable *via* other routes. The substitutions introduced in this investigation concerned only surface residues. The native overall conformation of cytochrome c was retained in all analogues. Apposite conclusions about the effects of

the pertinent substitutions on the electron transfer properties of cytochrome c can thus be made.

The activities of the acetylated cytochromes c towards cytochrome c oxidase (Figure 9) revealed that the positive charges at Lys^{72} and Lys^{73} in the native protein are about equally important for optimal electrostatic interaction with the oxidase, whilst the importance of a charge at Lys^{79} is smaller.

These results agree with several other recent studies with cytochrome c derivatives, modified at a *single* lysyl residue, and employed to define the binding domain for the oxidase. In these studies derivatives have been prepared by reacting cytochrome c with small amounts of ethyl thiotrifluoroacetate (Staudenmayer *et al*, 1976,1977), trifluoromethylphenylisocyanate (Smith *et al*, 1977,1980) or 4-chloro-3,5-dinitrobenzoate (Brautigan *et al*, 1978) to give mixtures of TFA-, TFC- and CDNP-derivatives of cytochrome c.

A large number of mono-substituted proteins have meanwhile been isolated from such mixtures by extensive ion exchange chromatography, *viz* 12 isomeric CDNP-derivatives (Osheroff *et al*, 1980) and 9 isomeric TFA-derivatives (Smith *et al*, 1980). Comparison of the reaction rates of those analogues have revealed the following orders of decreasing influence on the reaction with cytochrome c oxidase:

1. CDNP-derivatives: 13>72>86~87>8~27>73
2. TFA-derivatives: 13>72~25~87>8>79>27

These lysine residues are all situated on the front side (Swanson *et al*, 1977) and mainly on the top left part of the cytochrome c molecule; they surround the exposed heme edge (pyrrole ring II). The center of this domain is located near the β-carbon of Phe^{82} and contains the hydrophobic residues Ile^{81}-Phe-Ala^{83} (Ferguson-Miller *et al*, 1978).

A very similar concept of the binding site has been derived from a comparison of the chemical reactivity of the lysine side chains in free and oxidase-bound cytochrome c (Rieder and Bosshard, 1978a,b, 1980): lysyl residues 8, 13, 72 + 73, 86 and 87 were found to be most shielded in the complex; the chemical reactivity of lysyl residues 22, 39, 53, 55, 60, 99 and 100 was unaffected by complex formation, while that at positions 5, 7, 25, 27, 79 and 88 was somewhat less in the complexed molecule.

The activities of the mono-acetylated cytochrome c (Figure 9) not only confirm the occurrence of Lys^{72} in the interaction domain, but also

indicate that Lys^{73} lies in the contact area. The latter observation differs from the relatively small effect observed for the Lys^{73}-CDNP-derivative, as a result of which Lys^{73} was supposed to be in a position peripheral to the interaction domain (Osheroff *et al*, 1980). The difference may be due to a larger change in orientation of the Lys^{73} side chain in the CDNP derivative as compared to the acetyl-derivative.

The smaller effect on binding of the acetylated Lys^{79}-derivative suggests that this residue is situated in the periphery of the interaction domain, in agreement with the results from the TFA-derivatives and from the differential chemical modification method.

The relative importance of the three lysine residues is fully confirmed by the activities of the di-substituted cytochromes c (Figure 9). In summary, the lysyl residues 8, 13, 72, 73, 86 and 87 appear to line up the contact area between cytochrome c and the oxidase.

The center of the interaction domain is situated close to the point where the positive end of the dipole axis of cytochrome c- the estimated dipole moment of ferricytochrome c is 303 Debey - crosses the surface of the molecule (Koppenol *et al*, 1978). The negative end is near Lys^{99}, on the back of the molecule. Koppenol *et al* (1978) have calculated the electric potential field around cytochrome c and demonstrated that, at low ionic strength, the orientation of this field provides an explanation for the high yields found in reactions of the radicals e^-_{aq}, CO_2^- and O_2^- with ferricytochrome c.

Long range interactions due to the electric potential field imply that the protein approaches a negatively charged surface by directing its front side containing the exposed heme edge to that surface. This mechanism explains why a mixture of singly substituted, structurally isomeric, cytochromes c having the same net charge, can be separated on CM-cellulose at low ionic strength (Brautigan *et al*, 1978).

Since the binding site of cytochrome c for the oxidase is also involved in the binding of cytochrome c to cytochrome b_5 (Ng *et al*, 1977; Stonehuerner *et al*, 1979; Smith *et al*, 1980) and to cytochrome c peroxidase (Kang *et al*, 1978; Pettigraw, 1978) it seems that the dipolar charge distribution on cytochrome c is responsible for the primary interaction of the molecule with its enzymatic redox partners.

Recently it has been shown that the binding domain of cytochrome c

for cytochrome c_1, as determined with the isolated reductase complex (Speck *et al*, 1979), with succinate cytochrome c reductase (Ahmed *et al*, 1978; Smith *et al*, 1980) and with purified cytochrome c_1 (König *et al*, 1980a), virtually overlaps with that for the oxidase. The same result was obtained from the differential chemical reactivities of the lysine side-chains of cytochrome c in the presence and absence of cytochrome bc_1 or cytochrome c_1 (Rieder and Bosshard, 1978a,b, 1980). The similarity of the binding domains for various enzymes indicates the region of the exposed heme edge as the probable site of electron transfer.

6.5. Experimental

Materials, preparative and analytical methods used in this synthetic work have been described in section 4.4. The Appendix contains the TLC solvent systems, the reagents used to locate the peptide derivatives, and the abbreviations used.

Preparation of derivatives of lysine (Figure 2). Z-Lys(Ac)-OH (46): A mixture of lysine hydrochloride (9.1 g, 50 mmoles) and $CuSO_4 \cdot 5H_2O$ 6.25 g, 25 mmoles) in water (50 ml) was cooled to 0°, and the pH was adjusted to 10.5 with 4N NaOH. Acetic anhydride (6.0 ml, 62 mmoles) was added in portions with stirring while the pH was kept constant with a pH-stat loaded with 4N NaOH and the temperature maintained at 0°. The mixture was stirred for 1 h after completion of the addition. The precipitated, blue copper complex was filtered and washed thoroughly with water. The complex was subsequently dissolved in 1N HCl and H_2S was passed through the solution for 30 min with stirring.

After 2 h the precipitate of CuS was removed by filtration through a bed of hyflo. The colourless filtrate contained N^{ε}-acetyl-lysine (TLC: Rf=0.14 (C)), free of lysine. The pH of the solution was adjusted to 11.0 with 4N NaOH. Benzyloxycarbonyl chloride (8.0 ml) was then added in portions at 0° with vigorous agitation, while the pH was kept at 11.0 by addition of base. Stirring and cooling were continued for 6 hours whereon the resulting solution was extracted three times with ether (50 ml). The pH was then adjusted to approximately 1.5-2 with 1M $KHSO_4$ and the solution was extracted with ethyl acetate (3x100 ml). The combined extracts were washed successively with water and saturated NaCl solution, and dried (Na_2SO_4). Evaporation of the solvent gave 46 as a

colourless syrup (11.6 g, 72%), which was homogeneous by TLC (Rf=0.45 (B); 0.60 (C)); $|\alpha|_D^{22}$=-5.1° (c=3.0, methanol).
The product was characterized as the dicyclohexylamine salt, which was recrystallized from acetonitrile, m.p. 129.5-131°; $|\alpha|_D^{23}$=+8.5 (c=2.4, ethanol). Analysis: C, 66.85; H, 9.27; N, 8.31%. $C_{28}H_{45}N_3O_5$ (503.68) requires C, 66.76; H, 9.01; N, 8.34%. Lit: Benoiton (1963), m.p. 132-133°; $|\alpha|_D^{25}$=+8.6 (c=2, ethanol).
Z-Lys(Ac)-ONp (47) was prepared from 46 in DMF solution by treatment with p-nitrophenol and DCC in the usual manner. The product was recrystallized twice from propanol-2. Yield 74%; m.p. 117-119°; $|\alpha|_D^{20}$=-21.2° (c=1.3, acetone). Analysis: C, 59.31; H, 5.72; N, 9.41%. $C_{22}H_{25}N_3O_7$ (443.46) requires C, 59.59; H, 5.68; N, 9.48%.
Z-Lys(Ac)-OMe (48) was prepared in almost quantitative from 46 by treatment with diazomethane. It was crystallized from ether. TLC: Rf=0.66 (A); 0.70 (C); m.p. 87-89.5°; $|\alpha|_D^{23}$=-16.0° (c=1.09, methanol).
Z-Lys(Ac)-N_2H_3(49) was prepared by treatment of 48 with 2 equivalents of hydrazine ·hydrate in methanol solution. The product crystallized from the reaction mixture. Yield 88%; TLC: Rf=0.28 (A); 0.38 (C); m.p. 164.5-167.5°; $|\alpha|_D^{23}$=-9.8° (c=1.08, methanol). Analysis: C, 57.51; H, 7.52; N, 16.38%. $C_{16}H_{24}N_4O_4$ (336.384) requires C, 57.13; H, 7.19; N, 16.66%.
Z-Lys(Boc)-OH (50) was prepared by carbobenzoxylation of N^{ε}-Boc-Lysine (Schwyzer, 1961) as described for 46. The compound was obtained as a colourless oil in a 95% yield; TLC: Rf=0.21 (A); 0.78 (C); $|\alpha|_D^{24}$=-5.4° (c=1.14, methanol) (Lit: Wünsch *et al* (1966): $|\alpha|_D$=-5.87° (c=1, methanol)). and was characterized as the DCA-salt, prepared in ether: m.p. 144-146°; $|\alpha|_D^{25}$=+5.8° (c=0.97, methanol); =+7.7° (c=1, ethanol). Lit: Costopanagiotis *et al* (1968): m.p. 150-153°; $|\alpha|_D$=+5.0° (c=1, methanol); Wünsch and Zwick (1964): m.p. 156-157°; $|\alpha|_D$=+7.82° (c=1, ethanol).
Z-Lys(Boc)-OMe (51) was prepared from 50 by treatment with diazomethane in ether and was obtained in quantitative yield as an oil; TLC: Rf=0.80 (A); 0.89 (C); $|\alpha|_D^{25}$=-10.0° (c=1, acetone). Lit: Costopanagiotis *et al* (1968): m.p. 62-63°; $|\alpha|_D$=-10° (c=1, acetone).
Z-Lys(Boc)-ONp (52) was prepared from 50 in ethyl acetate solution by treatment with p-nitrophenol and DCC. It was recrystallized twice from propanol-2. M.p. 90-91.5°; $|\alpha|_D^{24}$=-16.9° (c=1.1, actone). Lit: Marchiori *et al* (1967): m.p. 88-90°; $|\alpha|_D$ =-16.5° (c=1.15, acetone).

Cytochrome c oxidase activity. Cytochrome c oxidase activities were determined using the ascorbate-TMPD system (Ferguson-Miller *et al*, 1976, 1978) by polarographic measurement of the rate of oxygen uptake at 25° with a Clark electrode mounted on a Gilson oxygraph.

Beef heart cytochrome c oxidase (ferrocytochrome c; oxygen oxidoreductase, EC 1.9.3.1) was a gift of Dr B.F. van Gelder and had been prepared as described (procedure III in: Hartzell *et al*, 1978).

Prior to enzymatic assay, samples of each cytochrome c analogue were rechromatographed on Sephadex G-25 columns (1.0 x 30 cm) using 25 mM Tris-acetate buffer, pH 7.8. For conditions of the assay see legend to Figure 9.

CHAPTER VII

SEMISYNTHETIC HSE^{65},LEU^{74}-CYTOCHROME C AND HSE^{65},LEU^{67}-CYTOCHROME C: PREPARATION AND PROPERTIES

The author is grateful to Herman H.K. Brinkhof, who performed the NMR experiments reported in this chapter.

Part of the results described here has been published:

-Boon, P.J., Tesser, G.I., Brinkhof, H.H.K. and Nivard, R.J.F. (1981) in 'Peptides 1980: Proceedings of the 16th European Peptide Symposium' (Brunfeldt, K., ed.) pp 428-434, Scriptor, Copenhagen.

-Boswell, A.P., Moore, G.R., Williams, R.J.P., Wallace, C.J.A., Boon, P.J., Nivard, R.J.F. and Tesser, G.I. (1981) Biochem. J. *193*, 493-502.

CHAPTER VII

SEMISYNTHETIC HSE^{65},LEU^{74}-CYTOCHROME C AND HSE^{65},LEU^{67}-CYTOCHROME C: PREPARATION AND PROPERTIES

7.1. Introduction

The critical step in the strategy developed for the semisynthesis of Hse^{65}-cytochrome c from three fragments is the conformationally guided formation of the Hse^{65}-Glu^{66} bond. Application of the scheme for the preparation of analogues of the protein will be limited to systems in which the alignment of complementary fragments 1-65 and 66-104 is not perturbed greatly. In the previous chapter it was shown that modifications, *i.e.* alterations of charge, at the surface of the protein do not interfere with the reaction. In order to determine the scope of the semisynthetic method it was decided to attempt the syntheses of cytochrome c analogues with a Tyr-Leu substitution at positions 67 and 74, respectively.

These tyrosyl residues are known to be almost invariant among eukaryotic cytochromes c; they have been replaced only rarely by other aromatic residues. Both residues form part of the 'left channel', the hydrophobic region at the left side of the protein, which extends from the surface to the heme moiety and also contains Trp^{59} (Dickerson and Timkovich, 1975). This particular region of the molecule was once thought to provide the pathway for the transport of the electron in the reduction step.

The presence of leucine in the bacterial *Paracoccus denitrificans* c_{550} at the position homologous to 74 was the main reason for abandoning this hypothesis. While Tyr^{74} is located near the surface of the molecule, Tyr^{67} is deeply buried within the heme crevice, close to Trp^{59}, which is also invariant. The three aromatic residues have been the target of earlier chemical modification studies on cytochrome c, generally resulting in molecules lacking a number of the native cytochrome c properties (for reviews on this subject see Dickerson and Timkovich (1975) and Ferguson-Miller *et al* (1979)). Iodination of Tyr^{74}, however, was later on shown not to affect the electron transfer properties of cyto-

chrome c (Osheroff *et al*, 1977).

The choice of leucine as the substituent for the tyrosyl residues was made since it seemed appropriate to maintain the hydrophobic character at the two sites in the protein.

The semisyntheses of Hse^{65},Leu^{74}- and Hse^{65},Leu^{67}-cytochrome c will be described in this chapter. In both syntheses the homoserine lactone-mediated coupling step proved to be as equally efficient as in the synthesis of the parent Hse^{65}-cytochrome c.

Some physicochemical properties of the two proteins and their reactivity towards cytochrome c oxidase have been determined, and will be described also. Finally, the results of a preliminary high resolution NMR study of the solution structure of the analogues will be presented, and the functional significance of aromaticity at positions 74 and 67 of cytochrome c will be discussed.

7.2. Semisynthesis of Hse^{65},Leu^{74}- and Hse^{65},Leu^{67}-cytochrome c

7.2.1. Synthesis of the Leu^{74}- and Leu^{67}-analogues of cytochrome c-(66-79)-tetradecapeptide

The strategy for the preparation of the two selectively protected cytochrome c sequences 66-79 with Tyr-Leu substitutions at positions 74 and 67, respectively, differed from the previously developed route in the order of assembly from the three subfragments. In the earlier strategy, which had evolved as a convenient adaptation of the route devised for the synthesis of the methionine containing sequence 66-80, the C-terminal fragment was the last to be introduced. A more direct approach, which is preferred because it obviates the conversion of the pentapeptide derivative 23 into the Boc-protected hydrazide 24 (Chapter VI, Scheme 3), has been followed here and is shown in Figure 1.

Two new peptide derivatives, the pentapeptide derivative 75 (as a free acid) and the protected hexapeptide derivative 79, were required.

Compound 75 was prepared from N^{α}-deprotected 17 which was acylated with Z-Leu-ONSu to give the crystalline tripeptide derivative 72. After liberation of the α-amino function in 72 by catalytic hydrogenation the free amine was condensed with the azide, prepared from 20. The resulting protected pentapeptide derivative 73, which readily crystallized from ethyl acetate in good yield (81%), was subsequently partially deprotect-

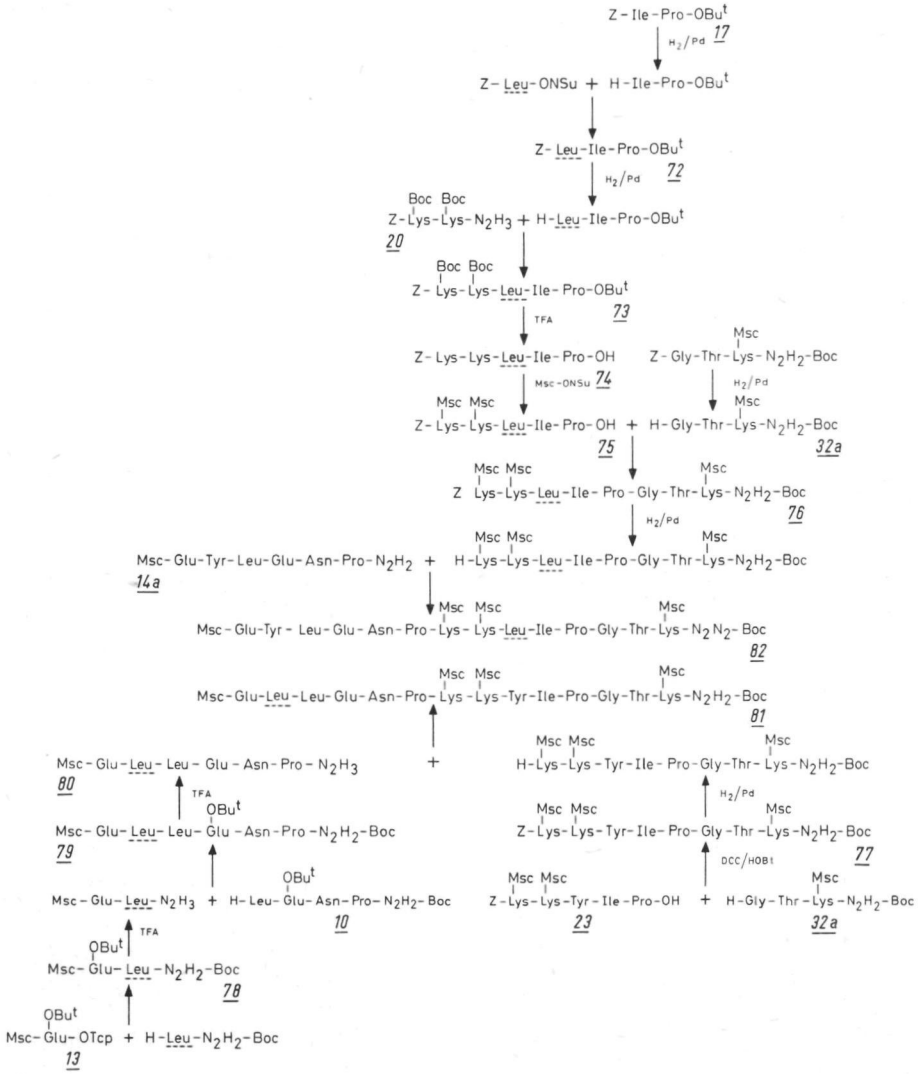


Fig 1 Scheme for the synthesis of Leu^{67}- and Leu^{74}-cytochrome c-(66-79)-tetradecapeptide derivatives 81 and 82.

ed in acid medium to give 74. Introduction of the two Msc-groups onto the lysyl side chains of 74 was performed with Msc-ONSu in the usual manner (cf preparation of 23, Chapter IV).

The hexapeptide derivative 79, containing the Tyr^{67}→Leu substitution was prepared by acylating the tetrapeptide derivative 10 with the product obtained from 78 by treatment with trifluoroacetic acid and conversion of the resulting partially protected dipeptide hydrazide into the corresponding azide. Compound 79 was isolated from the reaction mixture by an extraction with butanol-1. The remainder of the amino component 10 could be removed by use of Dowex-50W (H^+) ion exchange resin. The analytically pure, crystalline compound 78 was obtained by acylation of H-Leu-N_2H_2-Boc with the previously described, activated ester 13.

The octapeptide derivatives 77 and 76 were prepared from the appropriate fragments (Figure 1) by the method of König and Geiger (1970). These carbodiimide mediated condensations were sluggish at 0°, but proceeded readily at room temperature. The products were isolated by gel filtration on Sephadex LH-20 using DMF as the solvent. The yields of the analytically pure octapeptide derivatives varied between 65 and 75%.

Since the separation of the products from the respective pentapeptides was incomplete the isolated yield can presumably be raised considerably when a small excess of the amino component 32a is applied in the reactions in order to reach a quantitative conversion of the acid components 23 or 75. The hydrogenolytic removal of the benzyloxycarbonyl protecting groups in 76 and 77 was completed within 2 hours when carried out in methanol solution.

The final assembly of the tetradecapeptide derivatives 81 and 82 was achieved by N^{α}-deprotection of the two octapeptides 76 and 77, followed by condensation with the appropriate hexapeptide azides, prepared *in situ* from the peptide hydrazides 14a and 80. Isolation of the products 81 and 82 was again achieved by means of chromatography on Sephadex LH-20 in DMF. After a reaction period of 20 hours the reaction products were applied directly onto the gel filtration medium. The peptide derivative 81, representing the Leu^{67}-analogue of the native sequence, proved to be completely homogeneous by TLC (Rf=0.34(C)) and was obtained in a yield of 65%. Compound 82 (85%), representing the analogous Leu^{74}-sequence contained a trace of a more polar impurity as revealed by TLC,

but was used without further purification.

The hydrazide functions in 81 and 82 were liberated by treatment with 90% aqueous trifluoroacetic acid. Amino acid analyses of the desired 14-peptide hydrazides and of several intermediates are presented in Table 1. All analyses agree closely with the expected ratios for the amino acids.

Table 1 AMINO ACID COMPOSITION OF THE LEU^{74} AND LEU^{67} ANALOGUES OF THE SEQUENCE 66-104 AND OF SOME PEPTIDE INTERMEDIATES (Fig 1)

amino acid	76	77	79	81	82	66-104 Leu^{74}	66-104[1] Leu^{74}	66-104 Leu^{67}	66-104[1] Leu^{67}
Asp			0.97	0.97	0.99	3.03	3.03	3.05	3.10
Thr	0.97	0.99		1.00	0.89	3.19	3.10	2.84	2.96
Glu			2.00	2.03	2.04	5.38	4.12	5.03	4.34
Pro	0.99	0.93	0.96	1.87	1.95	n.d.	n.d.	1.98	1.70
Gly	1.00	1.04		1.05	0.95	2.10	2.10	2.06	2.01
Ala						3.05	2.95	2.95	2.94
Met						0.77	1.15	0.84	0.74
Ile	1.00	0.98		0.96	0.91	3.68	3.55	3.81	3.63
Leu	1.04		2.03	1.94	1.97	4.17	3.99	4.13	4.10
Tyr		0.92		0.89	0.97	1.67	0.33	1.90	0.20
Phe						0.83	0.88	0.99	0.89
Lys	2.94	3.07		3.15	3.04	8.16	0.56	7.78	<0.4
Arg						0.86	1.05	0.98	0.99

[1]analyses after treatment with FDNB.

7.2.2. Assembly of Hse^{65},Leu^{74}- and Hse^{65},Leu^{67}-cytochrome c

The covalent insertion of the synthetic 14-peptide derivatives, described in 7.2.1, between the isolated cytochrome c fragments 1-65 and 80-104 was performed using the methods developed previously.

The intermediate, semisynthetic sequences 66-104 were obtained by acylation of the N$^{\epsilon}$-Msc protected sequence 80-104 (compound IV, Chapter V) using a 50-70% excess of the azides prepared from 81 and 82.

The crude reaction products were isolated, after a reaction period of 40 hours, and then treated with alkali to remove the Msc-groups. The desired variants of the cytochrome c sequence 66-104 were separated by

gel filtration on Sephadex G-50 in 7% aqueous formic acid (Figure 2A).

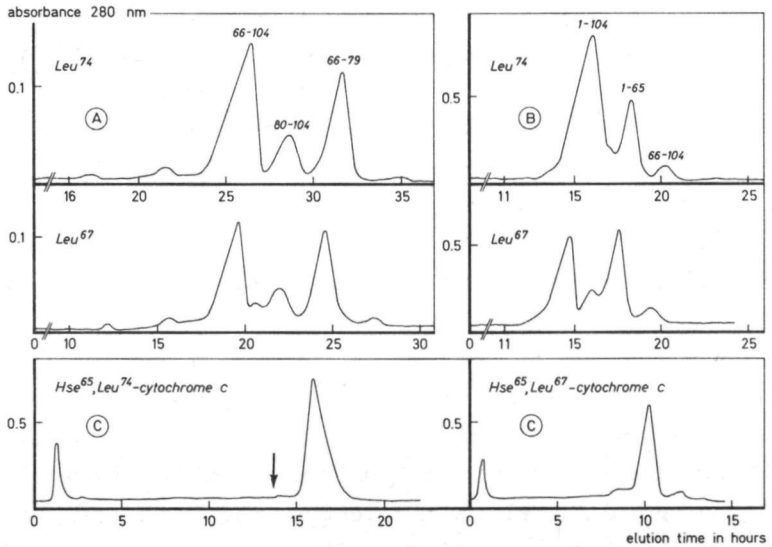


Fig 2 A: Isolation of semisynthetic cytochrome c-(66-104)-nonatriacontapeptides by gel filtration on Sephadex G-50 (2.5 x 120 cm) in 7% aqueous formic acid: Leu^{74}-analogue, flow rate: 8.5 ml/h;
Leu^{67}-analogue, flow rate: 11.0 ml/h

B: Isolation of semisynthetic cytochrome c analogues on Sephadex G-50 (2.5 x 120 cm) in 7% aqueous formic acid; flow rates: 11.0 ml/h

C: Purification of semisynthetic cytochrome c analogues on CM-cellulose (1 x 20 cm): Hse^{65},Leu^{74}-cytochrome c: elution was with 75 mM sodium phosphate buffer, pH 6.9 at arrow changed to 90 mM of the same buffer; flow rate: 18 ml/h
Hse^{65},Leu^{67}-cytochrome c: elution was with a linear gradient of sodium phosphate, 0.02 to 0.12M (2 x 150 ml), pH 6.9; flow rate: 24 ml/h
The leading peak in the chromatograms is due to ferricyanide.

The yields of the semisynthetic sequences were established by amino acid analyses, using norleucine as an internal standard, and were found to be 44% for the Leu^{74}-analogue and 42% for the Leu^{67}-analogue. The amino acid compositions of the 39-peptides were in good agreement with the theoretical figures, while similar analyses of FDNB-treated materials indicated that the deprotection step had been essentially complete (Table 1).

The crude 39-peptides have not been purified further; they were condensed, as such, with the heme peptide 1-65. The reactions were carried

out in 0.1M sodium phosphate buffer pH 7.0. The resulting cytochrome c analogues were isolated by gel filtration (Figure 2B).

Final purification was achieved, following renaturation in 4M urea, by chromatography on CM-cellulose. Representative elution profiles are reproduced in Figure 2C, which shows that both cytochrome c analogues were eluted as single entities. It must be noted that in both cases two additional products could be eluted from the columns by increasing the ionic strength of the phosphate buffer (not shown in Figure 2C).

The amount of these contaminants did not exceed, however, 5% of the applied material. Presumably these more positively charged species resulted from impurities in the semisynthetic peptides 66-104, similar to those that were shown previously to be present in the acetylated sequences 66-104 (see Figure 6 in Chapter VI). The yields of apparently homogeneous proteins were 53% for the Hse^{65},Leu^{74}- and 52% for the Hse^{65},Leu^{67}-cytochrome c; yields were determined spectrophotometrically assuming the same extinction coefficients for the analogues as for the native protein.

It is noteworthy that the syntheses of these analogues could well be performed on a considerably larger scale than that used previously in the preparations of the acetylated cytochromes c. Approximately 2 μmoles of both analogues have been prepared in one run, an amount sufficient (20-25 mg), but required, to carry out the NMR-studies described in section 7.5.

7.3. Physical properties

7.3.1. Hse^{65},Leu^{74}-cytochrome c

The UV-visible absorption spectrum of the *reduced* analogue is very similar to that of the native protein, with respect to the position of the absorption maxima at 315.5 nm (δ), 415.5 nm (Soret), 520 nm (β) and 550 nm (α), and their relative intensities (Table 2). The reduced analogue does not bind carbon monoxide.

The spectrum of the *oxidized* analogue shows the native absorption maxima at 280 nm, 290 nm, 409 nm (Soret) and 528 nm. The analogue also exhibits a band at 695 nm of normal shape, but with a lower intensity (89% at 25°) than the corresponding band in the native protein. With increasing temperature the 695 nm band decreases progressively in intensi-

Table 2 *COMPARISON OF SPECTRAL PROPERTIES OF NATIVE-, HSE^{65}, LEU^{74}- AND HSE^{65}, LEU^{67}-CYTOCHROME C*

	$A550_{red}/A280_{ox}$	$A415.5_{red}/A409_{ox}$	$A550_{red}/A520_{red}$	$A695_{ox}/A528_{ox}$
cytochrome c	1.25	1.22	1.75	13.3
Hse^{65},Leu^{74}-cytochrome c	1.28	1.22	1.74	14.8 14.1[a]
Hse^{65},Leu^{67}-cytochrome c	n.d.	1.20	1.66[b]	13.7

The spectra were recorded in 0.02M sodium phosphate, pH 7.0 at 20°;
a: measured in 0.2M phosphate, pH 7.0 at 20°.
b: ratio $A552_{red}/A520_{red}$

ty (Figure 3). At 55° the maximum disappears completely. The protein retains, however, a low spin configuration, since no absorption band at about 620 nm develops with the rise of temperature. Furthermore, the effects of temperature on the spectrum of the analogue are completely reversible. These observations can be interpreted as a displacement of the equilibrium between the isomer with the native His^{18}-Fe-Met^{80} ligation ('cold' isomer) and an isomer lacking the Met^{80}-heme iron bond ('hot' isomer), as described previously for the native protein (Schejter and George, 1964).

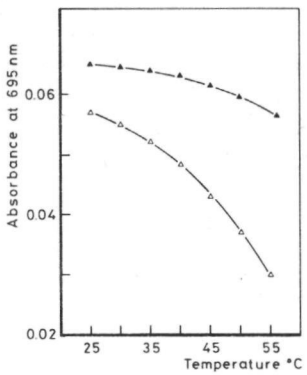


Fig 3 Changes in 695 nm absorption with temperature of cytochrome c (▲) and Hse^{65}, Leu^{74}-cytochrome c (△) in 20 mM sodium phosphate buffer, pH 6.9.

The equilibrium constants for this isomerisation were estimated from $K=A_t-A_{cold}/A_{hot}-A_t$, in which A_t is the absorbance at 695 nm at a particular temperature (Figure 3), A_{cold} is the absorbance of the protein with the native ligation (ε_{mM}=0.84 for cytochrome c, and assumed to be the same for

the analogue), and A_{hot} is the absorbance of the isomer lacking the Fe-S bond (ε_{mM}=0.38 for the native protein (Schejter and George, 1964); ε_{mM}= 0.25, estimated value for the analogue). The plots of log K against the reciprocal of the absolute temperature were satisfactorily linear between 25 and 50° for both proteins.

The standard enthalpy change for the interconversion of the two conformers was obtained by applying the integrated form of the van 't Hoff equation, ln K_{eq}=constant -$\Delta H/RT$, assuming that ΔH is temperature invarient over the temperature range considered. The thermodynamic parameters that govern the equilibrium between the 'cold' and the 'hot' isomer of Hse^{65},Leu^{74}-cytochrome c at 25° are compared with those for the native protein in Table 3.

Table 3 *THERMODYNAMIC PARAMETERS OF THE THERMAL TRANSITIONS OF NATIVE AND HSE^{65},LEU^{74}-CYTOCHROME C AT A TEMPERATURE OF 25°, DETERMINED FROM THE DATA IN FIGURE 3*

	ΔH, kcal/mole	ΔG, kcal/mole	ΔS, e.u.
cytochrome c	+14.6	+1.93	+42.5
	+14.6[a]	+1.7[a]	+43[a]
Hse^{65},Leu^{74}-cytochrome c	+12.0	+0.81	+37.5

a: data from Schejter and George (1964).

The values, which agree well for the native protein with those determined by Schejter and George (1964; see also Pettigrew *et al*, 1975a; Kaminsky *et al*, 1973), indicate that the enthalpy change for the disruption of the Met^{80}-iron co-ordination bond in Hse^{65},Leu^{74}-cytochrome c is less unfavourable than for the native protein, whilst the entropy change turns out to be less favourable. The net result is, however, a considerable decrease in the standard free energy change for the thermal transition: the equilibrium constant at 25° for the analogue is 0.25, which indicates that approximately 20% of the molecules is present in the ligand-displaced form. The corresponding figure for the native protein is approximately 3.5% (K=0.038).

Hse^{65},Leu^{74}-cytochrome c is completely (>98%) and rapidly reduced with ascorbate. The plot of the logarithm of the absorbance change (550

nm) versus time for the reduction is not linear (Figure 4), indicating a gradual decrease of the rate constant with time. This behaviour is ascribed to the presence of an appreciable amount of the 'hot' isomer of the analogue, which is expected not to be reducible by ascorbate.

Wilson and Greenwood (1971) have analyzed the kinetics of the reduction of ferricytochrome c by ascorbate, when an equilibrium distribution exists between a reducible (B) and a non-reducible conformer (A):

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} B \xrightarrow{k_2} C \qquad \text{(C=ferrocytochrome c)}$$

The observed rate of reduction of Hse^{65},Leu^{74}-cytochrome c (Figure 4) is to be expected only when the magnitude of the pseudo first order rate constant k_2 for the reduction is of comparable magnitude with the rate of isomerisation k_1.

It is important to note that Figure 4 indicates that the initial rate of reduction of Hse^{65},Leu^{74}-cytochrome c is nearly equal to that of the native protein.

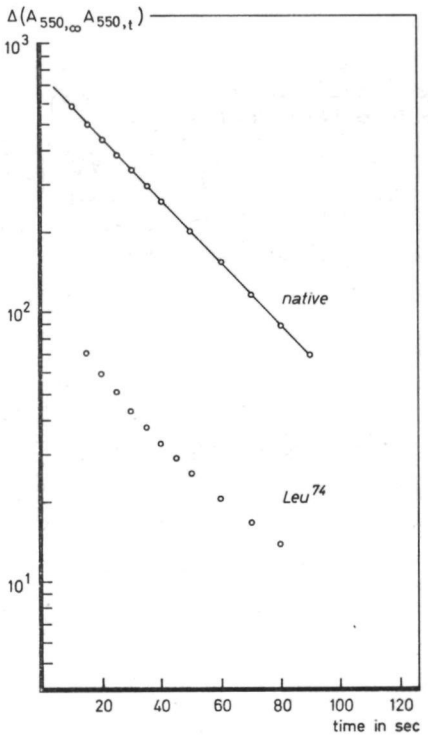


Fig 4 Half-logarithmic plot of the absorbance changes at 550 nm against time on reduction of cytochrome c and Hse^{65},Leu^{74}-cytochrome c with ascorbate under pseudo first order conditions. The absorbance is in arbitrary units.

7.3.2. Hse^{65},Leu^{67}-cytochrome c

The visible absorption spectrum of the ferric form of this analogue (Figure 5) shows a fully developed band at 695 nm, whilst the Soret band at 407.5 nm is blue-shifted compared to the absorption maximum of

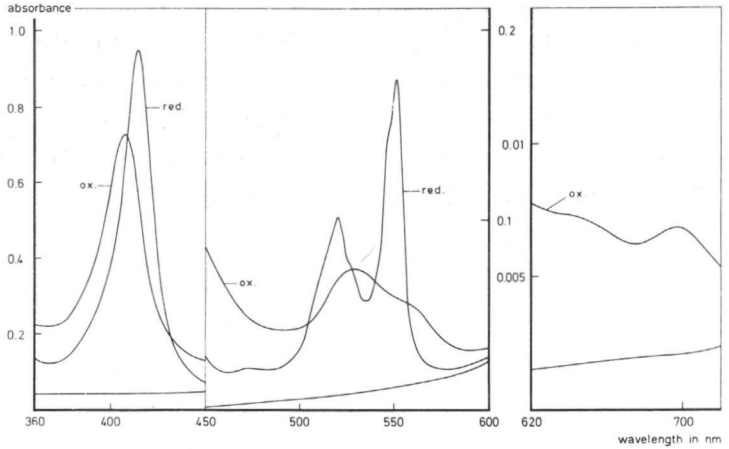


Fig 5 Visible absorption spectrum of the oxidized and reduced Hse^{65}, Leu^{67}-cytochrome c in 0.02M sodium phosphate buffer, pH 7.0 and 20°C.

the native protein (409 nm).

The spectrum of the *reduced* analogue exhibits the Soret band at 415.5 nm, the same value as found for the native protein. A striking difference is present around 550 nm: the α-band appears as a slightly split band, whose maximum is at 552 nm, with a shoulder at 548 nm; the extinction coefficient is 10-15% lower than that of the native protein. The other maxima in the absorption spectrum have nearly equal intensities as cytochrome c (Table 2).

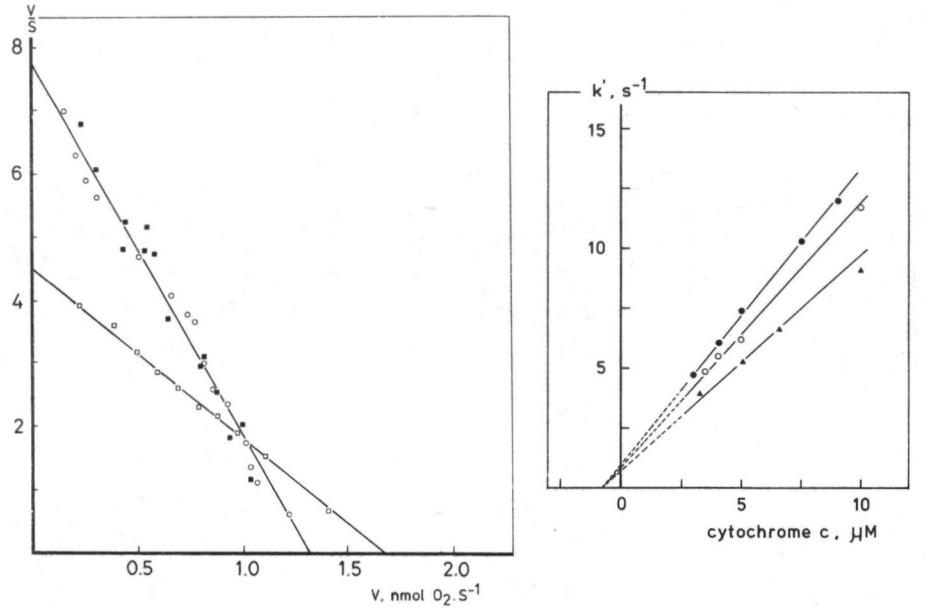
The analogue is completely and rapidly reduced by ascorbate. In contrast to the behaviour of the native protein and of the Hse^{65},Leu^{74}-analogue, the reduced Hse^{65},Leu^{67}-cytochrome c rapidly reacts with carbon monoxide.

The redox potential of the analogue, determined spectrophotometrically by equilibration with mixtures of ferri- and ferrocyanide of known potential at pH 7.0, was found to be +201±10 mV. A value of +272±10 mV was found for the native protein under identical conditions.

7.4. Biological activities

The steady state kinetics of the reactions of the two cytochrome c analogues with cytochrome c oxidase were investigated under conditions

that have been shown to be sensitive to changes in the binding of cytochrome c to the oxidase (cf Section 6.3.3). The results of the activity measurements of Hse^{65},Leu^{74}- and Hse^{65},Leu^{67}-cytochrome c, and the comparison with those of Hse^{65}-cytochrome c and the native protein are presented as Eadie-Hofstee plots in Figures 6A and 7. It is important to note that the single substitution of Met^{65} by Hse^{65} does not influence the activity.



A B

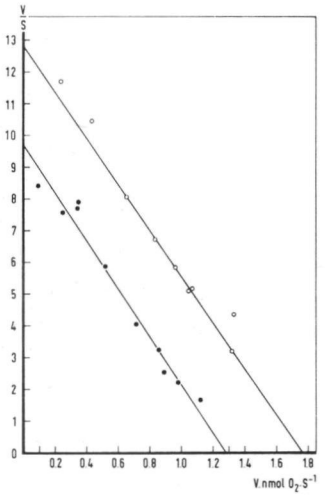
Fig 6A Steady-state cytochrome c oxidase activities of cytochrome c (o), Hse^{65}-cytochrome c (■) and Hse^{65},Leu^{74}-cytochrome c (□). V is in nmoles O_2 reduced/s and S is in μM cytochrome c. The assay medium, 25 mM Tris-acetate, pH 7.8 (1.25 ml), contained in addition to cytochrome c (0.01-1.0 μM): 250 mM sucrose, 0.1% tween-20, 20 mM ascorbate, 2 mM TMPD and 0.114 μM of a purified cytochrome c oxidase preparation

6B Relationship between the observed pseudo first order rate constants (k') and the concentrations of the oxidized native cytochrome c (o), Hse^{65},Leu^{74}-cytochrome c (●) and Hse^{65},Lys^{73}-acetyl-cytochrome c (▲). Ferrocytochrome c_1 was 1 μM in all experiments. Assay conditions: 10°C, 250 mM potassium phosphate, pH 7.0, 1% tween-20.

The substitution of Tyr^{74} by Leu leads apparently to a decrease in reactivity towards the oxidase at the very low cytochrome c concentrations

used; this indicates that the substitution interferes with the association to the oxidase. At higher concentrations the analogue appears to be as efficient a substrate as the native protein.

The ability of cytochrome c_1* to reduce cytochrome c does not seem to be affected by the substitutions in the Hse^{65},Leu^{74}-cytochrome c: the second order rate constant, $1.2\pm0.2\cdot10^6$ $M^{-1}\cdot s^{-1}$ for the latter compound, determined from the slope of the plot in Figure 6B, is equal to that found for the native protein, $1.1\pm0.2\cdot10^6$ $M^{-1}\cdot s^{-1}$. It should be noted, however, that the conditions used are rather insensitive for the detection of variation in the binding of cytochrome c to cytochrome c_1, since almost the same rate constant was measured for Hse^{65},Lys^{73}-Ac-cytochrome c, $0.9\pm0.2\cdot10^6$ $M^{-1}\cdot s^{-1}$.



The reactivity of Hse^{65},Leu^{67}-cytochrome c with cytochrome c oxidase appears to be remarkably enhanced over the whole concentration range measured. The virtually identical slopes of the Eadie-Hofstee plots in Figure 7 provide evidence that the ability of cytochrome c to bind to the oxidase is not affected by substitution of the internal Tyr^{67} by a residue of leucine. It is important to note that no autoxidation was detected under the assay conditions.

Fig 7 Steady state cytochrome c oxidase activities of cytochrome c (●) and Hse^{65}, Leu^{67}-cytochrome c (o) at low cytochrome c concentrations (0.01-1.0 μM). V is in nmoles O_2 reduced/s and S in μM cytochrome c. The assay medium, 25 mM Tris-acetate, pH 7.8 (1.65 ml), contained in addition to cytochrome c: 250 mM sucrose, 0.1% tween-20, 6.0 mM ascorbate, 1.2 mM TMPD and 0.145 μM of a purified cytochrome c oxidase preparation.

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*The author is grateful to Drs B.W. König and J. Wilms, who performed the experiments with their purified cytochrome c_1 (König *et al*, 1980a).

7.5. *Structural characterization of* Hse^{65},Leu^{74}- *and* Hse^{65},Leu^{67}-*cytochrome c*

7.5.1. *Introduction*

High resolution ^{1}H-NMR spectroscopy has now developed to the point where it can provide structural information about a protein in solution to a level of accuracy that is similar to that obtained from X-ray diffraction studies on crystalline proteins. Furthermore, information about the dynamics of the protein structure may be obtained by this technique. It was therefore decided to use NMR spectroscopy in an attempt to characterize possible structural alterations in cytochrome c on substitution of Tyr^{67} or Tyr^{74} by leucine. This kind of knowledge seemed to be a prerequisite for a meaningful interpretation of the changes in biological activity of these analogues.

Comprehensive accounts of the NMR-techniques that are currently used to study proteins have been given by Wüthrich (1976), Dwek (1977) and Campbell and Dobson (1979). The method with particular emphasis on its applicability to the study of cytochrome c has been reviewed (Levine *et al*, 1979).

The analysis of the NMR-spectrum of a protein proceeds in two stages: initially resonances are assigned to a special type of amino acid, and subsequently to a distinct amino acid residue of the protein sequence. The first stage assignment of resonances is usually carried out by double resonance techniques such as:

- *Spin decoupling*, to identify spin-spin coupled resonances
- *Cross-saturation*, which is observed in a system where nuclei are in slow exchange between different sites, such as in a mixture of ferri- and ferro-cytochrome c: saturation of the signal of a nucleus in ferri-cytochrome c results in a decrease in intensity of the corresponding signal in ferro-cytochrome c, and *vice versa*
- *Nuclear overhauser effect*, which is observed as a change in intensity of a resonance upon saturation of another resonance. The effect results from dipole-dipole interaction between nuclei, and its magnitude is therefore dependent on the separation between the nuclei
- *Multiple pulse sequences*, originally devised to measure relaxation times (Farrer and Becker, 1971), have been applied to simplify protein spectra: for example, in the spectrum obtained by the use of the

Carr-Purcell A sequence (CPA, 90°-τ-180°-τ) with τ=60 ms, the singlets and triplets with a coupling constant J of ca 8.5 Hz are in phase, while the doublets are 180° out of phase. The combination of this multiplet selection method with double irradiation, *spin-echo double resonance*, often proves to be valuable to identify spin-spin coupled resonances (Campbell *et al*, 1975; Campbell and Dobson, 1975).

The subsequent, second stage assignment will then provide the structural information on the protein. In favourable cases unambiguous assignments can be made from a comparison of spectra of the protein from various species or of the chemically modified proteins. However, the procedures to assign resonances to distinct amino acid residues are not usually straightforward, since the mechanisms by which a resonance is shifted from its primary position, *i.e.* the chemical shift of a particular proton in a random coil protein, are not understood fully. In a diamagnetic protein most secondary shifts result largely from the ring-current field produced by aromatic groups, whereas the shielding caused by carbonyl groups, and effects dependent upon the degree of ionization of ionisable groups and hydrogen bonding, usually contribute much less.

In addition to information from intrinsic probe effects, identification of resonances of a particular amino acid may result from the information gained when a protein spectrum is deliberately perturbed, *e.g.* by the binding of protons, the binding of a diamagnetic ligand or of a paramagnetic species (Campbell and Dobson, 1979). Frequently, however, a knowledge of the X-ray structure is required to arrive at an unambiguous assignment.

Cytochrome c has been studied intensively by NMR spectroscopy because of its relatively small size, the knowledge of its crystal structure and its occurence in two stable oxidation states. The resonances of the heme group and those of the axial ligands, which experience very large secondary shifts, were the first to be assigned (Keller and Wüthrich, 1978, and references cited therein). The most detailed NMR study of cytochrome c has been carried out by Moore and Williams (1980a-f).

In a recent series of papers they give assignments of virtually all aromatic proton resonances as well as resonances of a number of aliphatic amino acids of both the ferric and ferrous form of the horse protein. It was concluded that cytochrome c exhibits a single, well-ordered struc-

ture in both oxidation states, which is rigidly constructed in the interior and more flexible at the surface. Thus the aromatic side chains of Tyr^{48} and Phe^{46}, at close proximity in the molecule, and Phe^{10} were found to be considerably restricted in their mobility, whilst Trp^{59} is completely immobile. Conversely, the region of the protein close to Ile^{57} was found to be relatively flexible in both oxidation states.

It was further concluded from comparison of the spectra of ferricytochrome c, ferrocytochrome c and cobalticytochrome c that the conformation in the region of Ile^{57} and Tyr^{74} was different for ferri- and ferrocytochrome c. This important observation provides a structural basis for the differences in chemical and physical properties of ferri- and ferrocytochrome c. It also demonstrates the power of the NMR method, since X-ray diffraction studies had not established such differences.

In the following comparison of the spectra of the semisynthetic cytochrome c analogues with that of the native protein special reference will be made to the studies of Moore and Williams, referred to above. In Table 4 the assignments of resonances of reduced Hse^{65}-, Hse^{65},Leu^{74}- and Hse^{65},Leu^{67}-cytochrome c, made so far, are compared with those of the native protein. Resonance designates are those introduced by Moore and Williams (1980a,b) for ferrocytochrome c: assigned non-heme resonances in the aromatic region are designated A1...An starting with the furthest downfield resonance, assigned non-heme methyl resonances are designated M1...Mn starting with the furthest upfield methyl resonance.

The analysis of the spectrum of each analogue will be described separately in the following sections.

7.5.2. *Hse^{65}-cytochrome c*

Since all analogues of cytochrome c, described here contain the Met^{65}-Hse^{65} substitution, it was essential to determine any change in structure due to this modification.

In Figure 8 the spectrum of ferro-Hse^{65}-cytochrome c is compared with that of the native protein. The spectra are very similar, which indicates that the modification of Met^{65} has not seriously perturbed the structure of the protein. The resonances most affected by the modification are those of Trp^{59} (A5, A24, A30), the *meta* proton resonance of Tyr^{74} (A26) and the δ-CH_3 resonance of Ile^{57} (M4), which is shifted upfield

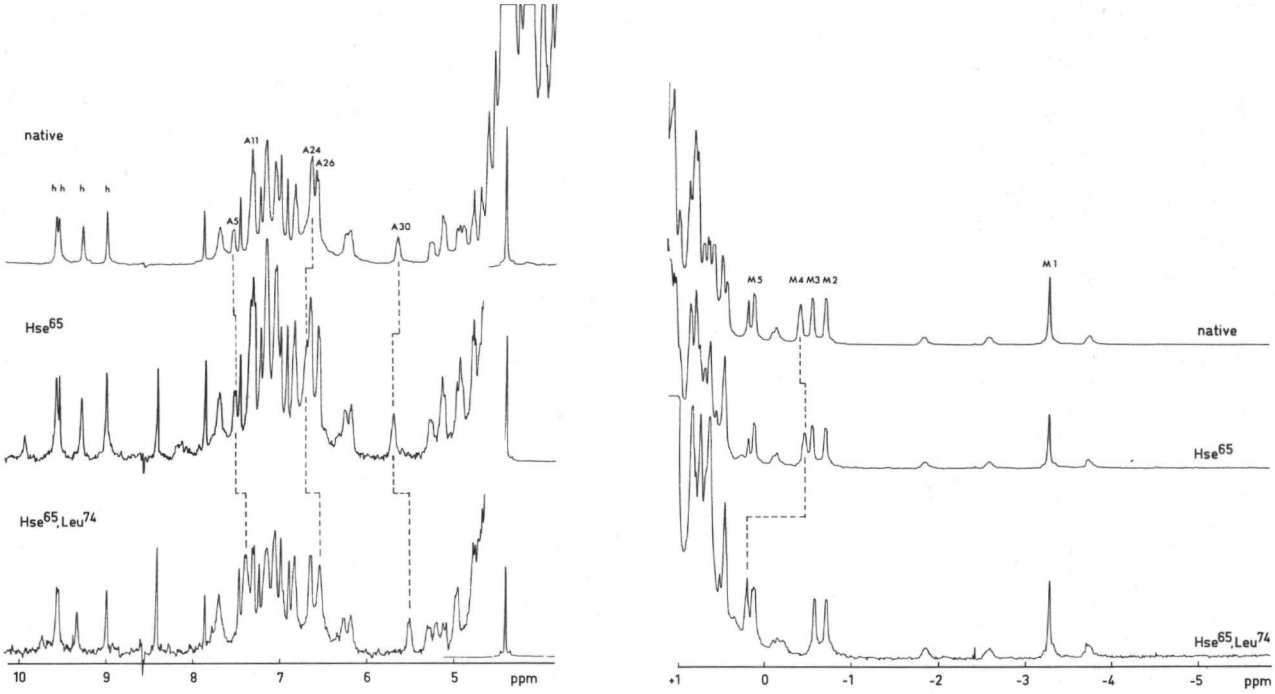

Fig 8 The aromatic and upfield aliphatic window regions of conventional 360 MHz spectra of ferrocytochrome c, ferro-Hse^{65}-cytochrome c and ferro-Hse^{65},Leu^{74}-cytochrome c in 0.1M deuterated sodium phosphate buffer at pH 6.8 and 57^{o}C. Differences in three of the Trp^{59}resonances, and of the Ile^{57}-δCH_3resonance M4 are indicated; h, heme meso-CH resonances.

Table 4 COMPARISON OF NMR SPECTRA OF FERROCYTOCHROME C, FERRO-HSE^{65}-CYTOCHROME C, FERRO-HSE^{65}, LEU^{67}-CYTOCHROME C AND FERRO-HSE^{65}, LEU^{74}-CYTOCHROME C [1]

	resonance designate	native ppm	Hse^{65}- ppm	Hse^{65},Leu^{67}- ppm	Hse^{65},Leu^{74}- ppm
Heme c [2]					
meso CH α		9.32		9.22	9.36
meso CH β		9.59		9.45	9.57
meso CH γ		9.62		9.40	9.59
meso CH δ		9.04		8.78	9.02
thioether bridge 2 CH		5.27			5.30
CH_3		1.53			1.55
thioether bridge 4 CH		6.34		6.25	6.32
CH_3		2.57			2.59
ring methyl 1		3.49			3.50
ring methyl 3		3.88			3.89
ring methyl 5		3.60			3.60
ring methyl 8		2.21			2.13
Aliphatic region					
Met^{80} SCH_3	M1	-3.28	-3.28	-3.15	-3.29
CH	C1	-3.73	-3.73	-3.75	-3.73
CH	C2	-2.58	-2.58	-2.68	-2.58
CH	C3	-1.87	-1.87	-1.84	-1.88
Leu^{32} CH_3	M2	-0.76	-0.76	-0.77	-0.75
CH_3	M3	-0.60	-0.60	-0.62	-0.61
Ile^{57}	M4	-0.43	-0.48	-0.44	0.15
not assigned	M5	0.07	0.07	0.05	0.09
not assigned	M6	0.38	0.42	0.39	0.39
not assigned	M7	0.43	0.43	0.39	0.42
His^{18} C2	A32	0.50		0.40	0.47
His^{18} C4	A33	0.13		0.01	0.10
Aromatic region					
His^{26} C2	A8	7.52			7.52
His^{26} C4	A18	7.06			7.05
Trp^{59} C2	A19	6.99	6.98		6.95
Trp^{59} C4	A5	7.58	7.57	7.70	7.46
Trp^{59} C5	A24	6.68	6.70	7.07	6.61
Trp^{59} C6	A30	5.76	5.78	6.88	5.59
Trp^{59} C7	A16	7.07	7.10	7.33	7.04
Phe^{10} *ortho*	A17	7.10[a]	7.11[a]	7.11[c]	7.12[a]
Phe^{10} *meta*	A25	6.70[a]	6.70[a]	6.68[c]	6.61[a]
Phe^{10} *para*	A27	6.34	6.25	6.25	6.26
Phe^{36} *ortho*	A9	7.40	7.36	7.34	7.37
Phe^{36} *meta*	A20	6.87	6.88	6.88	6.89
Phe^{36} *para*	A15	7.1	7.11	7.07	
Phe^{46} *ortho*	A4	7.75[a]	7.74[a]	7.63	7.74[a]
Phe^{46} *meta*	A17	7.22[a]	7.20[a]	7.15	7.20[a]
Phe^{46} *para*	A3	7.75	7.74	7.72	7.74
Phe^{82} *ortho*	A23	6.71	6.72	6.83	6.70
Phe^{82} *meta*	A10	7.40	7.39	7.38	7.42
Phe^{82} *para*	A13	7.2	7.2	7.2	7.21
Tyr^{48} AC	A21	6.78[a]	6.83[a]	6.83[c]	6.84[a]
Tyr^{48} BD	A29	6.18[a]	6.14[a]	6.15[c]	6.16[a]
Tyr^{48} A	A14	7.19[b]	7.19[b]	7.18[b]	7.19[b]
Tyr^{48} B	A22	6.78[b]	6.80[b]	6.83[b]	6.85[b]
Tyr^{48} C	A28	6.33[b]	6.30[b]	6.32[b]	6.27[b]
Tyr^{48} D	A31	5.59[b]	5.59[b]	5.57[b]	5.59[b]
Tyr^{74} *ortho*	A11	7.22	7.20	6.99	not present
Tyr^{74} *meta*	A26	6.64	6.60	6.71	not present

[1] Chemical shift values were measured at pH 6.8 and at 57°, unless indicated otherwise; Hse^{65}, Leu^{67}-cytochrome c was studied at pH 5-6.
Resonance assignments and resonance designates for the native protein are those given by G.R. Moore and R.J.P. Williams (1980a,b), Eur. J. Biochem. *103*, 493-502 and 503-512, and Boswell *et al* (1980b), J. Inorg. Biochem. *13*, 347-353.

[2] Assignment of the heme c resonances of the native- and the Hse^{65},Leu^{74}-protein were carried out by G.R. Moore (The Inorganic Chemistry Laboratory, Oxford, U.K.) *via* measurements of nuclear Overhauser Effects (cf Keller and Wüthrich, 1978).

[a] Measured at 97°

[b] Measured at 15°

[c] Measured at 77°

by the ring-current fields of Tyr^{74} and Trp^{59}. Similar effects were seen in the spectrum of tuna ferrocytochrome c on carboxymethylation of Met^{65} (Boswell *et al*, 1981). All pertinent resonances belong to residues located in the conformationally sensitive region around Ile^{57}. The following explanation given by Boswell *et al* (1981) accounts for the observed differences.

Since the hydroxyl function of Tyr^{74} is probably hydrogen-bonded to the carboxyl function of Glu^{66}, any perturbation that affects Glu^{66}, *e.g.* the Met^{65}-Hse^{65} substitution in this case, may also affect Tyr^{74}; a change in orientation of Tyr^{74} will result in a change in chemical shift of M4 (Ile^{57}). The shift of the Trp^{59} resonances may be associated with the same structural perturbation. Since the Trp^{59} resonances will experience only a small ring-current effect from Tyr^{74}, Boswell *et al* suggest that the shifts may be caused by a change in the Trp^{59}-heme orientation. The analysis of the spectrum of Hse^{65},Tyr^{67}-cytochrome c, described below (Section 7.5.4.), reveals, however, that the secondary shift of the Trp^{59} resonances arise mainly from the ring-current field of Tyr^{67}. Therefore, a small readjustment of the side chain of Tyr^{67}, which is situated close to Tyr^{74}, could equally well explain the observations.

Collectively, the observed shifts in the spectrum of Hse^{65}-cytochrome c, which are very small (<0.06 ppm) and do not exceed the differences between horse and tuna ferrocytochrome c (Moore and Williams, 1980e), point to a slight rearrangement on the left hand side of the protein. In agreement with this conclusion it has been shown that Hse^{65}-cytochrome c and the native protein have different affinities for the rabbit anti-horse cytochrome c anti-sera directed against the Ile^{57}-containing determinant (Barstow *et al*, 1977; Berman and Harbury, 1980). The minor nature of the structural perturbation is indicated further by the unchanged biological properties of Hse^{65}-cytochrome c (Figure 6).

The NMR spectrum of ferri-Hse^{65}-cytochrome c has not been studied in detail. It is notable, however, that the heme methyl resonances and the Met^{80} resonances, both experiencing very large paramagnetic shifts, are not perturbed significantly. This observation agrees with the unchanged 695 nm band in the visible absorption spectrum and indicates that the coordination centre in Hse^{65}-cytochrome c has not been disturbed by the

Met^{65}-Hse^{65} exchange.

7.5.3. Hse^{65},Leu^{74}-cytochrome c

The comparison of the spectrum of Hse^{65},Leu^{74}-cytochrome c with that of the Hse^{65}-protein reveals virtually unchanged signals for the Met^{80} and the heme meso protons, and a close similarity for the aromatic regions of the spectra (Figure 8). The slight perturbations in the aromatic region of the spectrum due to the Tyr^{74}→Leu substitution were analyzed by carrying out resonance assignments. These were established by homonuclear decoupling experiments.

The identification of the Trp^{59} signals is shown as an example in Figure 9: irradiation of the triplet at 6.61 ppm (A24) decouples a triplet at 5.59 ppm (A30) and a doublet at 7.44 ppm (A5) leaving a doublet and a singlet, respectively; irradiation of the triplet at 5.59 ppm causes decoupling of the triplet at 6.61 ppm and a doublet at 7.04 ppm (A16) giving a doublet and singlet, respectively. The decoupling of A24 (6.61

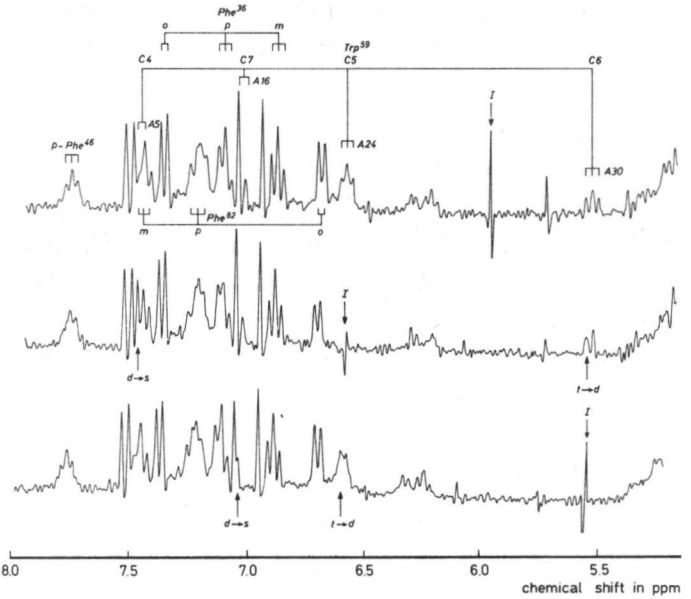

Fig 9 Aromatic region of the 270 MHz convolution difference spectrum of Hse^{65},Leu^{74}-cytochrome c at pH 6.8 and 57°C. Two of the decoupling experiments that provided the assignment of the Trp^{59} resonances are shown. First and second stage assignments are indicated (*cf* Table 4).

ppm) to a doublet is somewhat obscured by the presence of the exchange-broadened meta resonance of Phe^{10}; the resonances of this residue sharpen at higher temperature. The identification of the four, spin coupled, resonances affords directly the assignment of the single Trp^{59} residue in the analogue.

Similar experiments were done to identify other resonances in the aromatic area of the spectrum. Since the first stage assignments appeared to be almost identical to those of Hse^{65}-cytochrome c, the second stage assignments, collected in Table 4, followed from the direct comparison. A major difference with Hse^{65}-cytochrome c lies in the resonance at 6.61 ppm, which is a triplet of the Trp^{59}-C5 proton in the Leu^{74}-protein, while the corresponding resonance in the spectra of Hse^{65}- and native cytochrome c is a doublet (A26) coupled to another doublet at 7.22 ppm (A11).

It is clear that these latter resonances must belong to Tyr^{74}, since they are absent in the spectrum of the Leu^{74}-protein. The assignments of the resonances of the reduced Hse^{65},Leu^{74}-cytochrome c (Table 4) reveal that, in addition to the lack of the two tyrosine doublets, the Trp^{59} resonances are the only aromatic signals which have been perturbed to a significant extent. The observed shifts may be caused in part by the disappearance of the ring-current field of Tyr^{74} or by a slight reorientation of the aromatic ring of Tyr^{67} (see Discussion).

NOE experiments, carried out by G.R. Moore, have provided the complete set of resonance assignments of the heme moiety (Table 4). The only heme resonance that proved to be perturbed significantly in the Hse^{65},Leu^{74}-analogue is that belonging to the methyl group at position 8 on ring IV, which lies in the current fields of Trp^{59} and Tyr^{67} (Perkins, 1980).

In the aliphatic region (Figure 8) of the spectrum a dramatic shift is observed for the resonance M4, assigned to Ile^{57} δ-CH_3 (Moore and Williams, 1980b,c,d). The large upfield shift of this resonance in the native protein is caused by the ring current fields of Trp^{59} and Tyr^{74} (Perkins, 1980). The removal of Tyr^{74} in the analogue thus causes the anticipated effect, thereby firmly establishing the assignment of M4.

It is of interest that the temperature dependence of M4 in Hse^{65}, Leu^{74}-cytochrome c is much less than in either the native protein or

Hse^{65}-cytochrome c. The observed shifts, 0.12 ppm (27°), 0.15 ppm (57°) and 0.18 ppm (77°), are not greater than for most other methyl resonances (*e.g.* M2, M3, M5 and M6; Moore and Williams, 1980c). The temperature dependence of M4 in the native protein has been interpreted as being due to the flexibility of the region near Ile^{57}, in which either Ile^{57} or Tyr^{74}, or both, move with respect to each other. The mobility can now be characterized more precisely as a movement of the Tyr^{74} side chain, while the position of Ile^{57} in the molecule, more specifically its position relative to Trp^{59}, seems to be fixed.

The stability of reduced Hse^{65},Leu^{74}-cytochrome c is comparable with that of the native protein; spectra were recorded up to 97° without denaturation of the protein and without a change in oxidation state.

In conclusion, the solution structure of the *reduced* Hse^{65},Leu^{74}-protein proves to be nearly identical to that of the reduced native protein. The observed differences can be ascribed to the local change introduced at the surface of the molecule.

The *oxidized* protein has not been studied in the same detail as the reduced form. It is noteworthy, however, that the increased thermolability of the analogue (see Section 7.3.1) is also reflected in the temperature dependent behaviour of the Met^{80} and heme resonance. Thus, the CH_3-singlet of Met^{80}, which resonates at -23.4 ppm (25°, pH 7.0) in the analogue (native protein: -23.8 ppm) broadens and shifts downfield with increasing temperature; at 55° the resonance becomes an almost unobservable signal at -18.6 ppm. The corresponding resonance in the native protein on the other hand, shifts to -20.4 ppm without appreciable broadening. The singlet resonances at 32.6 ppm and 35.4 ppm, assigned to the methyl substituents of pyrrole rings II and IV, respectively (Keller and Wüthrich, 1978; native protein 32.8 and 35.4 ppm), also broaden with temperature, concomitantly with the appearance of new resonances at about 22.5 and 20.0 ppm above 40°.

These changes prove to be completely reversible and are similar to those observed for native cytochrome c, above pH 9 (Gupta and Koenig, 1971), or when the heme region is disrupted by the addition of methanol to the ferric protein (Boswell *et al*, 1980a) or by other methods (Morishima *et al*, 1977).

These observations parallel the effects of temperature on the 695 nm

absorption band (see Section 7.3.1) and are characteristic for a shift in the equilibrium between the native structure and the high pH form of ferricytochrome c, in which the heme ligand is displaced with retainment of a low spin configuration of the heme iron.

7.5.4. Hse^{65},Leu^{67}-cytochrome c

The NMR spectrum of reduced Hse^{65},Leu^{67}-cytochrome c was studied up to 77°, at which temperature the derivative undergoes slow, irreversible denaturation. In the spectrum obtained at 57° (Figure 10) a close similarity with the spectrum of Hse^{65}-cytochrome c (Figure 8) is directly apparent from the aliphatic region; the resonances of Met^{80} (M1), Leu^{32} (M2 and M3), M5 and M6 (not firmly assigned, Moore and Williams, 1980b), and, most notably, of Ile^{57} (M4) are nearly unchanged. From these observations it may be concluded that the conformation of the analogue must resemble that of the native protein.

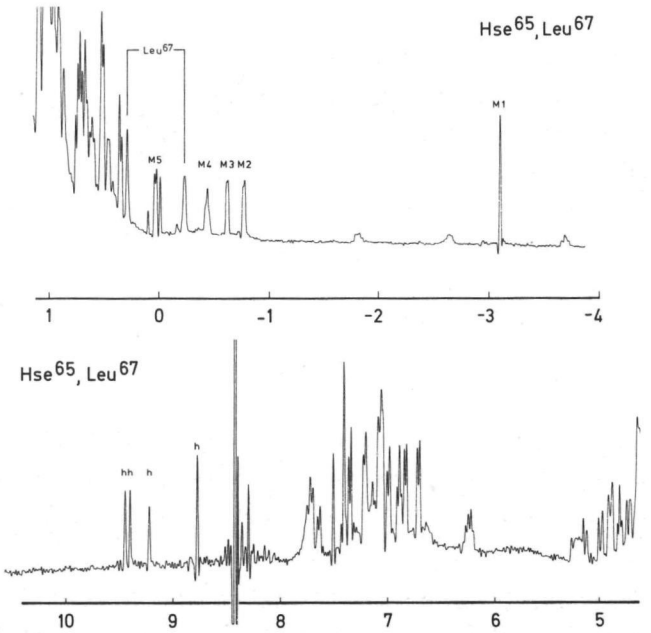

Fig 10 The aromatic and upfield aliphatic window regions of the resolution enhanced (sine bell) 360 MHz spectrum of Hse^{65},Leu^{67}-cytochrome c in 0.1M deuterated sodium phosphate buffer, pH about 5.5, at 57°C; h, heme meso CH resonances.

The aromatic region of the spectrum does not immediately reveal a close similarity. However, further analysis proved that the perturbation of this region is only due to the absence of Tyr^{67}.

First stage assignments for all the resonances in the aromatic region were obtained by double resonance techniques. The identification of the Trp^{59} resonances, which proved to be critical, is presented in Figure 11: irradiation of the triplet at 6.88 ppm (A30) converts a triplet at 7.07 ppm (A24) and a doublet at 7.33 ppm (A16) into a doublet and a singlet, respectively, while irradiation of the triplet at 7.07 ppm causes the collapse of the triplet at 6.88 ppm (A30) and the doublet at 7.70 ppm (A5) into a doublet and a singlet, respectively.

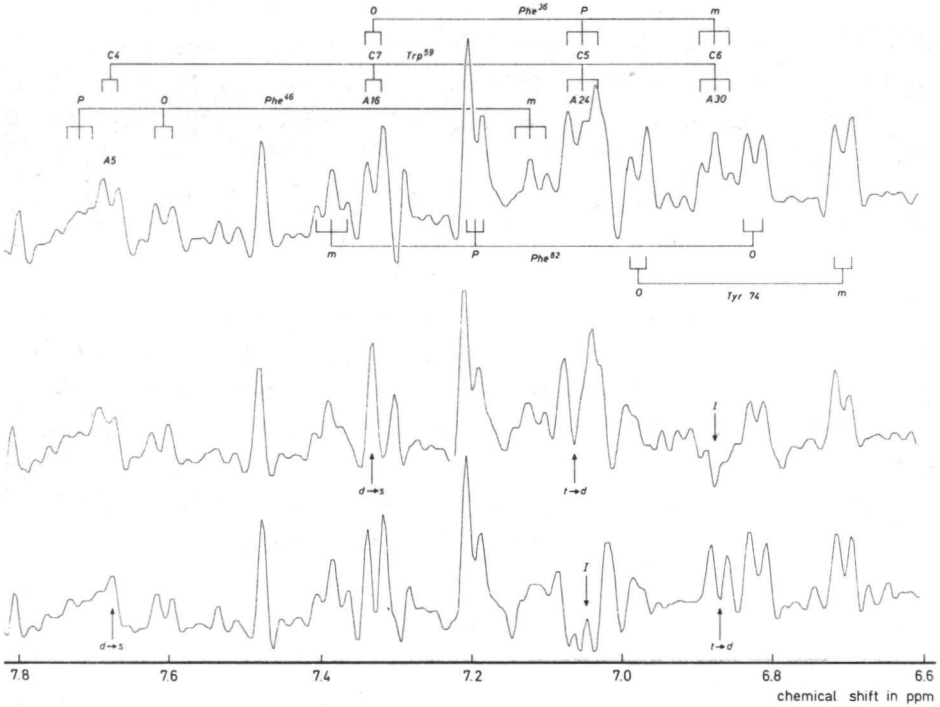

Fig 11 Aromatic regions of the resolution enhanced 360 MHz spectra of Hse^{65},Leu^{67}-cytochrome c at pH 5-6 and at 57°C. Two of the decoupling experiments that provided the assignment of the Trp^{59} resonances are shown. First and second stage assignments are indicated (cf Table 4).

The second stage assignments (Table 4 and Figure 11) for Phe^{46}, Phe^{82}, Phe^{10} and Tyr^{48} were based on the correspondence of their resonances with those of Hse^{65}-cytochrome c. The three resonances of Phe^{46} were observed separately at 57°, albeit with relatively low intensity. With increasing temperature these resonances gained in intensity, whilst the *ortho* doublet shifted to the resonance position of the *para* triplet (7.72 ppm).

By comparison, the Phe^{46} resonances of the native protein were assigned at 87°, where the *ortho* and *para* resonances coincided at 7.75 ppm (Moore and Williams, 1980a). The temperature dependence of the Tyr^{48} resonances was found to be very similar to that in the native protein. The behaviour of the Phe^{46} as well as the Tyr^{48} resonances, which show slow exchange at temperatures below 37° and approach fast exchange at higher temperature, indicate that the bottom part of the molecule exhibits a native-like motility (cf Campbell *et al*, 1976; Moore and Williams, 1980a,c).

The assignment of the coupled doublets at 6.99 and 6.71 ppm to Tyr^{74} follows from the fact that it is the only remaining tyrosine expected to give an AABB-type spectrum. The assignment of the Phe^{36} resonances was based on the consideration that no significant differences in chemical shifts, as compared with Hse^{65}-cytochrome c, were to be expected for this peripheral residue, as was found for most other aromatic resonances. The Phe^{36} resonances happen to coincide with three of the resonances of Trp^{59}.

Support for this conclusion was obtained from the small effect on the Trp^{59} triplet at 7.07 (A24) ppm when irradiating the coupled doublet at 7.70 ppm (A5). The presence of the two proton Phe^{36} triplet (A15) at the very same position explains that observation.

A comparison of the assigned resonances for Hse^{65},Leu^{67}-cytochrome c with those of Hse^{65}-cytochrome c (Table 4) reveals that the only resonances showing substantial differences in chemical shifts upon substituting Tyr^{67} by Leu are those belonging to Trp^{59} and Tyr^{74}. The removal of the ring-current field of Tyr^{67} largely explains the observed shift differences. Strong support for this explanation comes from a comparison (Table 5) of the observed shift differences with those recently calculated for the effect of the Tyr^{67} ring current field on the Trp^{59}

and Tyr^{74} resonances (Perkins, 1980).

Table 5 COMPARISON BETWEEN OBSERVED AND CALCULATED (Perkins, 1980) CONTRIBUTIONS OF TYR^{67} TO THE SECONDARY CHEMICAL SHIFT OF TRP^{59} AND TYR^{74} RESONANCES

assignment	chemical shift in ppm		observed effect in ppm	calculated effect in ppm
	native	Leu^{67}		
Trp^{59} C4	7.60	7.72	+0.12	+0.15
Trp^{59} C7	7.10	7.33	+0.23	+0.26
Trp^{59} C2	6.98	7.07	+0.09	<0.1
Trp^{59} C5	6.70	7.05	+0.35	+0.52
Trp^{59} C6	5.74	6.88	+1.14	+1.15
Tyr^{74} *ortho*	7.22	6.99	-0.23	-0.19
Tyr^{74} *meta*	6.67	6.71	+0.04	<0.1

The remarkable agreement between the calculated and observed values also furnishes, reliable assignments for the individual Trp^{59} resonances C4, C5, C6 and C7, which were hitherto unknown.

The temperature dependence of the resonances in the aliphatic region, illustrated in Figure 12, shows that only resonance M4, the Ile^{57} δCH_3 shifts strongly back to its primary position. The magnitude of this shift is virtually identical with that experienced by the native M4 resonance. Furthermore, at all temperatures M4 is found at almost the same resonance position as in the native protein. This similarity indicates that the structure of the leftside of Hse^{65},Leu^{67}-cytochrome c, *i.e.* the relative positions in space of Trp^{59},Tyr^{74} and Ile^{57}, is very similar to that in the native protein.

The additional resonances at -0.22 and +0.33 ppm (Figure 12a) are assigned tentatively to the methyl groups of Leu^{67}. The magnitude of the secondary shifts experienced by these methyl groups is about 1.1 and 0.6 ppm, respectively. The similarity in structure between the analogue and the native protein suggests that the Tyr^{67} resonances in the spectrum of the native protein will experience a comparable secondary shift. The resonances of Tyr^{67} have not been so far identified in the aromatic region, however. This has been ascribed tentatively

to a large effect of the heme ring current field, which should shift these resonances into the aliphatic region (Moore and Williams, 1980a).

The observation that the intensities of the Leu^{67} methyl resonances in a Carr Purcell A spectrum (Figure 12e) are very low, possibly due to a very short spin-spin relaxation time (T2), suggests a restricted mobility of the Leu^{67} side chain. The absence of resonances of Tyr^{67} in spectra of the native protein is likely therefore to be due to exchange broadening.

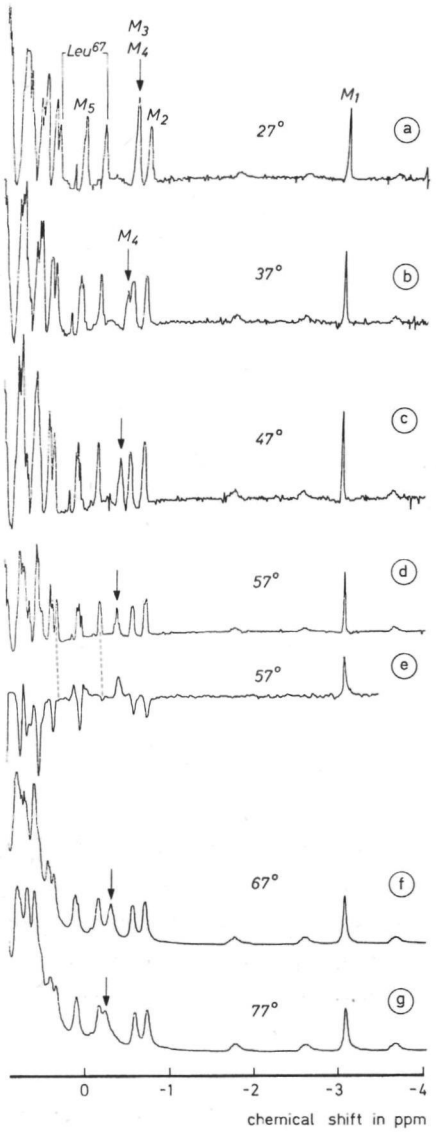

Fig 12 The upfield window region of convolution difference (a-d) and conventional (f and g) 270 MHz spectra of Hse^{65},Leu^{67}-cytochrome c at ca pH 6 and at various temperatures. (e): Carr-Purcell A spectrum with τ=60 ms. The temperature dependence of the Ile^{57}-δCH_3 resonance M4 is indicated by the arrows.

7.6. Discussion

The successful semisynthesis of the two cytochrome c analogues described in this chapter indicates that the semisynthetic approach studied in this thesis may be of greater value in elucidating structure-function relationships of the heme protein than originally anticipated. Whilst repacement of Tyr^{74}, a residue situated close to the surface of the cytochrome c molecule, by Leu^{74} could be expected not to disrupt the native, spatial arrangement in the complex 1-65: 66-104, it was surprising to find that the analogous replacement of Tyr^{67}, a residue deeply buried within the heme crevice, did not do so either. Both conformationally guided, homoserine lactone mediated, coupling reaction proved to be very efficient processes and yielded the pure analogues in yields of 50-53%.

The effects of the incorporation of leucine in either position 74 or 67 on the physical and enzymatic properties are quite different. The most significant difference in properties of Hse^{65},Leu^{74}-cytochrome c compared to the native protein is the considerably decreased thermostability of the oxidized form, which exists at 25°C for approximately 20% in the 'hot' conformer, lacking the native Fe-S coordination. From the experiments described there were no indications that the reduced analogue is less thermally stable than ferrocytochrome c.

A decreased reactivity towards cytochrome c oxidase was apparent at low ionic strength of the medium and at low concentrations of the protein. At higher concentrations the activity was comparable to that of the native protein. These observations indicate a decreased binding affinity of the analogue for the oxidase. However, the conclusion does not seem to be as unambiguous as for the N^{ϵ}-acylated analogues, described in the previous chapter, which show similar effects. It can not be ruled out that the ferric analogue resulting from oxidation of the analogue by cytochrome c oxidase is again partly present as the 'hot' conformer, which may be not reducible by the ascorbate-TMPD system. Dissociation of the cytochrome c-cytochrome c oxidase complex would then be an obligatory step and this would explain the observed steady state kinetics.

The rate constant of the reaction of Hse^{65},Leu^{74}-cytochrome c with purified cytochrome c_1 proved to be virtually the same as for the na-

tive protein (Figure 6B). The reaction was studied at 10° and at high ionic strength, conditions which make the measurement rather insensitive to changes in binding of cytochrome c to the enzyme since the second order rate constant found for the Lys^{73}-acetyl analogue is of comparable magnitude. However, conditions have been described recently for the reaction of cytochrome c with purified cytochrome c_1 (König *et al*, 1980a,b), with cytochrome c reductase and the cytochrome bc_1 complex (Speck *et al*, 1979; Ahmed *et al*, 1978), which do reveal relatively large differences in rate for cytochrome c analogues modified at position 73.

In conclusion it appears that the enzymatic properties of cytochrome c are not influenced to a major degree by the substitution of Tyr^{74} by Leu^{74}.

The near constancy of a tyrosine residue at the homologous position 74 in the known eukaryotic sequences - only 2 out of 90 species show a conservative replacement by phenylalanine (Dickerson and Timkovich, 1975) - and the increased thermolability observed on substituting Tyr^{74} in the horse protein by leucine, suggests that a tyrosine residue is best suited to provide the 'left channel' of the eukaryotic proteins with the necessary rigidity. In most prokaryotic sequences known to date, the homologous position is occupied by penylalanine, while valine is found in the bacterial *Rhodomicrobium vanniellii* c_2 and leucine in *Paracoccus denitrificans* c_{550}.

In the latter protein, 134 amino acids in length, the stability may be retained by the addition of an extra loop of amino acids in the region of the leucine (Timkovich and Dickerson, 1976). The much smaller respiratory cytochrome c_{551} of *Pseudomonas aeruginosa*, with 82 amino acids, might retain its rigidity by accommodating two additional residues of proline in its left side sequence (Almassy and Dickerson, 1978).

The properties of the Hse^{65},Leu^{74}-cytochrome c provide strong evidence against a specific functional role of an aromatic group in the spatial position 74 in the electron transfer event itself, as proposed in the earlier Dickerson-Winfield mechanism (Dickerson, 1974; Takano *et al*, 1973).

The preliminary characterization of the Hse^{65},Leu^{67}-cytochrome c has revealed some features that seem to be unique among the known chemically modified cytochromes c.

For the interpretation of those properties it is an essential finding that the structure of the analogue in solution is very similar to that of the native cytochrome c. This follows from the NMR-study and from the observation that the analogue binds to the oxidase equally well as the native protein (Figure 7).

As a consequence it seems permissable to attribute the following properties to the local substitution $Tyr^{67} \rightarrow Leu$:

1. the shift of the α-band absorption maximum from 550 to 552 nm with the appearance of a shoulder at 548 nm
2. the reduction of the mid-point redox potential (Em,7) by about 70 mV
3. the considerably increased reactivity with cytochrome c oxidase.

The slightly split α-band in the visible spectrum of the reduced analogue has been observed in only a few bacterial cytochromes c and has even been proposed as an additional criterion for classification of bacterial cytochromes c (Dickerson and Timkovich, 1975).

The phenomenon is not very well understood, but has been viewed as an indication of some peculiar arrangement of the iron ligands. The present NMR-study of Hse^{65},Leu^{67}-cytochrome c does not give any indication for a different orientation of the iron ligands His^{18} or Met^{80}, in the analogue. On the other hand it is apparent from the resonance assignments in Table 4 that those nuclei which are bound or in very close proximity to the heme group, experience small, but significant, chemical shift differences as compared to the native protein. These differences cannot readily be ascribed to the removal of the ring current field of Tyr^{67} as such, since similar effects are also found at several positions in the protein which are far removed from that locus. The nuclei effected are the four meso heme protons, His^{18}-C2 and C4, Met^{80}-CH_3 and β-CH, and the thioether bridge 4 CH. This suggests that the ring current field of the heme group has been perturbed in the analogue, or in other words it is indicative of a redistribution of the π-electron density in the porphyrin moiety.

The shift of the α-band in the absorption spectrum, indicating some variation in the excitation of delocalized π-electrons to unoccupied levels of the heme system, is probably a further reflection of that change. The direction of the shift, a red shift, indicates a slight enlargement of the delocalisation of electrons in the porphyrin system.

The concomitant appearance of the shoulder at 548 nm, indicates a lowering of the symmetry of the π-system. It would be interesting to ascertain whether these spectral changes are correlated to the second deviation introduced by Leu^{67}, *viz* the decrease of the mid-point redox potential by ca 70 mV.

The elucidation of the structural factors that are responsible for the inconstancy of the redox potential, varying between 100 and 400 mV for the large number of known cytochromes c, all retaining the same overall folding pattern, is fundamental to an understanding of the mechanism of electron transfer in cytochrome c. Various attempts have been made to determine those factors. It has been suggested (Kassner, 1972, 1973) that the dielectric constant of the heme environment plays a major role: a more hydrophilic heme environment stabilizes the charged ferric state better than a hydrophobic environment and should result in a lower redox potential.

A consequence of this suggestion should be that some correlation exists between the redox potential and the degree of exposure of the heme group. Comparison of the X-ray structures of *Pseudomonas aeruginosa* cytochrome c_{551} and *Chlorobium thiosulfatophilum* cytochrome c_{555} reveals, however, that their heme groups are exposed equally to the medium, while their redox potentials are quite different: 285 mV *vs* 145 mV, respectively (Korszun and Salemme, 1977). It has also been shown (Fiechtner and Kassner, 1978) that the heme in *Prosthecochloris aestuarii* cytochrome c_{555}, a small cytochrome c with a redox potential of 103 mV, is less exposed than the heme group in horse cytochrome c. These examples demonstrate that the degree of exposure of the heme does not correlate with the redox potential.

The importance of the packing of amino acid side chains around the heme to the redox potential has been stressed (Salemme, 1977). Comparative studies on the redox potentials of c_2 cytochromes from photosynthetic bacteria have indicated that in several cases amino acid substitutions are consistent with changes in hydrophobicity near the heme being at least a partial determinant of the redox potential (Pettigrew *et al*, 1975b, 1978).

These studies also provided indications for the possible involvement of the heme propionate residues in the pH dependence of the redox po-

tentials of some c_2 cytochromes. Recently, the change in redox potential with pH of *Pseudomonas aeruginosa* c_{551} was studied by Moore *et al* (1980). A transition was observed having a pK_a-value in the physiological pH range (6.2-7.3). It was characterized by a decrease in redox potential from about 300 to 235 mV and a shift of the α-peak maximum from 551 to 553 nm with increasing pH. Simultaneously the α-peak becomes asymmetrical. It was found by monitoring the transition by NMR that one of the propionic acid residues of the heme was the ionisable group.

The heme group in Hse^{65},Leu^{67}-cytochrome c appears to be similarly exposed as in the native horse protein as revealed by the NMR-study and the hydrophobicity around the heme is increased rather than decreased by the Tyr^{67}-Leu substitution. The observed decrease in the mid-point potential of about 70 mV in addition to the spectral changes in the α-band region, parallels the changes described above for *pseudomonas aeruginosa* c_{551}.

A possible explanation for the deviating properties is offered by the recent comparison of the X-ray structures of tuna ferrocytochrome c and ferricytochrome c, which have been refined independently at high resolution (1.5 Å and 1.8 Å; Takano and Dickerson, 1980).

It was found that tyrosine-67, besides being hydrogen bonded to the Met^{80}-sulfur atom, provides a hydrogen bond to a buried water molecule, which is held in place by additional hydrogen bonds provided by the side chains of Asn^{52} and Thr^{78}. Thr^{78} itself, is also hydrogen bonded to the outer propionic acid residue of the heme ring III. The conformational difference between the ferric and ferrous forms of cytochrome c appeared to be mainly a shift of this buried water molecule over a distance of about 1Å, accompanied by movement of the Tyr^{67} and Asn^{52} side chains. The hydroxyl group of Thr^{78} appeared, however, to remain in the same position. In the oxidized state, the water molecule is closer to the heme and the heme has moved 0.15 Å out of its heme crevice. The changes lead to a more polar microenvironment for the heme.

The main effect of removing Tyr^{67} and replacing it by a residue of leucine might well be the disturbance of this delicate network of hydrogen bonding. Presumably the hydrogen bonds remaining in the analogue, *viz* those between Asn^{52}-H_2O-Thr^{78}-outer propionic acid, might be less

effective in depressing the dissociation of the outer propionic acid residue, which has an intrinsically low pK_a of about 4.5-5. This would result in a partial negative charge on the heme carboxyl group or, less dramatically, in a change in distribution of charge within the hydrogen-bonded system. The higher charge at the outer propionic acid would stabilize the ferric form of the analogue providing a plausible explanation for the reduction in redox potential. Similarly it would also explain the increase in delocalisation of the π-electron system of the porphyrin group as well as the introduction of asymmetry within that system, features believed to be connected with the spectral changes in the α-band region.

In this view the higher rate of reaction of Hse^{65},Leu^{67}-cytochrome c with cytochrome c oxidase is an expected result since the reduced analogue, is internally destablished leading to a lower barrier for the transfer of an electron to the oxidase. Furthermore, the properties of Hse^{65},Leu^{67}-cytochrome c provide strong evidence against the direct involvement of aromaticity at position 67 in cytochrome c in the electron transport. To summarize, a functional role for Tyr^{67} in cytochrome c is suggested, in that its presence and positioning in the molecule may contribute to the control over the redox properties by stabilizing the inner part of the heme crevice.

It is noteworthy to add that Myer *et al* (1979), when studying the dependence of the redox potential of cytochrome c on denaturation of the protein with urea, concluded that the maintenance of the integrity of the tryptophan-heme domain of the crevice, *i.e.* its deepest part, was the most important factor. The 2.7 Å structure of *Chlorobium thiosulfatophilum* c_{555}, a low potential cytochrome c (+145 mV) exhibiting a slightly split α-band, was shown to maintain the same overall folding pattern seen in eukaryotic cytochromes c (Korszun and Salemme, 1977). Since the available data indicate that this protein does not contain an aromatic residue in a position structurally homologous to Tyr^{67}, its absence may very well be the main structural factor responsible for the properties of c_{555}.

There is one further observation made during the analysis of the NMR spectra of ferro-Hse^{65},Leu^{67}-cytochrome c which merits comment, since it provides information about the conformational difference be-

tween ferri- and ferrocytochrome c (Moore and Williams, 1980d). It was shown in Section 7.5.4 and Table 5 that the side chain of Tyr^{67} is the main source of the secondary shifts that are experienced by the Trp^{59} protons.

Conversely, it is known (Moore and Williams, 1980a) that there is a large difference in chemical shift of the Trp^{59} resonances, especially of the triplet resonance A3O, between the ferric and ferrous form of the protein. The same authors have shown that the Trp^{59} resonances of the oxidized protein do not exhibit a significant temperature dependence (Moore and Williams, 1980d), as would be the case when the paramagnetism of the heme was responsible for the secondary shifts. It is suggested therefore that the differences in chemical shifts of the Trp^{59} resonances in the two redox states of cytochrome c are the result of a change in orientation of the Tyr^{67} side chain within the protein. This finding implies a re-appraisal of the conformational difference, defined by Moore and Williams as a readjustment of the region on the left side surface of the protein including Ile^{57} and Tyr^{74}. The data above suggests that the deeper part of the heme crevice is involved also in the conformational change.

This observation constitutes an independent observation in solution of a feature which was seen by Takano and Dickerson (1980) as one of the largest differences between the X-ray structures of ferri- and ferrocytochrome c, and provides a firmer basis to that difference.

7.7. *Experimental*

For general experimental methods see Chapter IV, Section 4.4. Abbreviations and solvent systems used in thin layer chromatography are listed in the Appendix.

Z-Leu-Ile-Pro-OBu^t (72): Compound 17 (2.05 g, 4.90 mmoles) was hydrogenated in methanol (20 ml) as described earlier (Chapter IV) to give H-Ile-Pro-OBu^t as a colourless syrup. A solution of this amino component in DMF (20 ml) was treated at 0° with HOBt (0.82 g, 5.3 mmoles) and with Z-Leu-ONSu (1.60 g, 4.41 mmoles; crystalline, from propanol-2/ether/pet. ether (1:3:2), 84% yield, m.p. 112.5-114°; lit. 51%, m.p. 116-117° (Anderson *et al*, 1964). The mixture was maintained at room temperature for 24 hours, and the product was isolated following the

usual work-up procedure in ethyl acetate. Crystallization from ethyl acetate-ether mixtures afforded several crops of compound 72 as long, thin needles (1.72 g, 74%), melting at 106-107°; $|\alpha|_D^{22}$=-97.5° (c=1.9; methanol); TLC: Rf=0.82 (B), 0.89 (C). Analysis: C, 66.07; H, 8.72; N, 8.03%. $C_{29}H_{45}N_3O_6$ (531.69) requires C, 65.60; H, 8.53; N, 7.90%.

Z-Lys(Boc)-Lys(Boc)-Leu-Ile-Pro-OBu^t (73): Compound 72 (1.00 g, 1.88 mmoles) in methanol (15 ml) was hydrogenated for 10 min in the presence of Pd/C as the catalyst. The resulting solution was filtered, and the solvent was evaporated *in vacuo* to give H-Leu-Ile-Pro-OBu^t as a clear syrup (Rf=0.50 (B)), which when evaporated from ethyl acetate solution crystallised spontaneously as needles. A solution of the amino component in DMF (2.5 ml) was combined at 0° with the azide that was freshly prepared from Z-Lys(Boc)-Lys(Boc)-N_2H_3 (20, 2.25 g, 2.00 mmoles; *cf* preparation of 21) in DMF (15 ml). The mixture was kept at 0°, and at pH 8, for 15 hours. The solvents were then evaporated *in vacuo* to give a thick oil, which solidified on treatment with ethyl acetate. The solids were dissolved in chloroform and the solution was washed with water. The organic layer was dried and then evaporated to give pure 73 as an oil, which could be crystallized from ethyl acetate. Yield: 1.51 g (81%), m.p. 196-197°, $|\alpha|_D^{22}$=-76.5° (c=0.95; methanol); TLC: Rf=0.78 (B), 0.94 (C). Analysis: C, 62.32; H, 8.81; N, 9.94%. $C_{51}H_{85}N_7O_{12}$ (988.28) requires C, 61.98; H, 8.67; N, 9.92%.

Z-Lys(Msc)-Lys(Msc)-Leu-Ile-Pro-OH (75): The removal of the acid labile protective functions from the pentapeptide ester 73 by treatment with 90% aqueous trifluoroacetic acid and the subsequent protection of the liberated N^{ε}-lysyl groups in 74 to give 75, were performed as described earlier in Chapter IV, for the preparation of the analogous pentapeptide 23 from 21. Compound 75 was obtained in a yield of 85%. The amorphous solid did not have a well defined melting point (75-85°, dec); $|\alpha|_D^{22}$=-60.5° (c=0.95; methanol); TLC: Rf=0.62 (C, trace of an impurity at 0.80), 0.40 (B). Analysis: C, 51.82; H, 7.03; N, 9.42%. $C_{45}H_{73}N_7O_{16}S_2 \cdot H_2O$ (1050.26) requires C, 51.45; H, 7.20; N, 9.34%.

Z-Lys(Msc)-Lys(Msc)-Leu-Ile-Pro-Gly-Thr-Lys(Msc)-N_2H_2-Boc (76): A solution of the pentapeptide derivative 75 (0.194 g, 0.188 mmoles) and the tripeptide derivative 32a (hydrochloride, 0.114 g, 0.188 mmoles) in DMF (3.0 ml) was treated with ethyldiisoproplylamine (33 µl; 1 equiva-

lent at 0°, followed succesively by HOBt (50.8 mg, 0.376 mmoles) and DCC (40.5 mg, 0.197 mmoles). The mixture was stirred for 30 min at -10° and then kept at room temperature for 20 hours. The amino component had reacted completely during this time. The precipitate of DCU was collected by centrifugation, and the resulting clear solution was applied to a column of Sephadex LH-20 (2.5 x 120 cm), pre-equilibrated with DMF. The column was then developed with DMF. Since the leading peak in the elution profile contained a shoulder a the low molecular weight side, the corresponding eluates were pooled to give two fractions. Each was evaporated to dryness and the residues were dissolved in methanol (1-2 ml). The products were precipitated by addition to stirred, cold ether (20 ml), collected by centrifugation, washed with ether and dried *in vacuo* over KOH. The main fraction (190 mg, 63,8%) contained the almost pure octapeptide derivative 76; the minor fraction (54.5 mg, 18%) was contaminated with unreacted 75. TLC (system B) on compound 76 indicated a single component (Rf=0.31), whilst in system C (Rf=0.49) a trace of an unidentified impurity (Rf=0.38) was evident. M.p.: 80-90° (slow decomposition); $|\alpha|_D^{22}$=-52.0° (c=0.56; methanol). Analysis: C, 49.51, H, 7.11; N, 11.32; S, 5.72%. $C_{66}H_{111}N_{13}O_{25}S_3 \cdot H_2O$ (1600.90) requires C, 49.52; H, 7.11; N, 11.37; S, 6.02%. Amino acid analysis: see Table 1.

Z-Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro-Gly-Thr-Lys(Msc)-N_2H_2-Boc (77): The preparation of this compound from compound 23 (Chapter IV) and compound 32a and its subsequent purification were achieved as described above for compound 76. Yield: 70.4%; m.p.: >123° (dec); $|\alpha|_D^{22}$=-36.5° (c=0.51; methanol); TLC: Rf=0.47 (C). Analysis: C, 50.72; H, 6.97; N, 10.91%. $C_{69}H_{109}N_{13}O_{26}S_3$ (1632.88) requires C, 50.75; H, 6.73; N, 11.15%. Amino acid analysis: see Table 1.

Msc-Glu(OBu^t)-Leu-N_2H_2-Boc (78): The protected hydrazide H-Leu-N_2H_2-Boc (0.90 g, 3.75 mmoles) in DMF (13 ml) was treated with compound 13 (Chapter IV, 2.0 g, 3.75 mmoles) at -10° for 1 hour and a further hour at 0°. The usual work-up procedure gave a syrup which was freed from residual trichlorophenol by triturating with diisopropyl ether. Crystallization from ethyl acetate-ether afforded 84% (1.83 g) of pure 78 (as the monohydrate), m.p. 82-84°. $|\alpha|_D^{22}$=-40.6° (c=0.89; methanol); TLC: Rf=0.65 (B); 0.79 (C). Analysis: C, 49.57, H, 7.67; N, 9.65%. $C_{24}H_{43}N_4O_9S \cdot H_2O$ (581.71) requires C, 49.55; H, 7.80; N, 9.63%.

Msc-Glu-Leu-Leu-Glu(OBu^t)-Asn-Pro-N_2H_2-Boc (79): Compound 78 was treated with 90% trifluoroacetic acid for 45 min at room temperature. The amorphous Msc-Glu-Leu-N_2H_3 was isolated by ether precipitation (92%; m.p. >50° (slow decomposition); $|\alpha|_D^{22}$=-31.4° (c=1.11, methanol)). The free hydrazide (69 mg, 0.13 mmoles) in DMF (0,3 ml) was converted into the azide using the *in situ* method and then combined with a solution of H-Leu-Glu(OBu^t)-Asn-Pro-N_2H_2-Boc (10), freshly prepared by catalytic hydrogenation of 9 (100 mg, 1.27 mmoles), in DMF (0.8 ml).

The reaction was allowed to proceed at 0° for 72 hours. The solvents were then removed *in vacuo* and the resulting residue was dissolved in butanol-1. The solution was extracted with 1M $KHSO_4$ (2x) and subsequently washed with water until neutral. Evaporation of the solvents gave an amorphous solid, on trituration with ethyl acetate (99 mg, 74%). A small amount of the amino component 10 was removed with the aid of Dowex-50W H^+-ion exchange resin as described for compound 14. The product, which still contained a trace of an impurity (Rf=0.58 (C)), was isolated by precipitation from a methanol solution by addition of ether. M.p.: 186-188° ($|\alpha|_D^{22}$=-85.4° (c=0.70; methanol); TLC: Rf=0.26 (B); 0.69 (C). Amino acid analysis: see Table 1.

NMR spectroscopy

360 MHz proton NMR spectra were recorded on a Bruker 360 MHz spectrometer operating in the Fourier transform mode. The spectra given in Figure 12 were obtained using a 270 MHz spectrometer (Inorganic Chemistry Laboratory, University of Oxford). The H_2O resonance was used as an internal reference; spectra were subsequently referred to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS), taking the temperature dependent shift of the H_2O resonance into account. Chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of DSS. In some experiments the spectral resolution was enhanced by applying the sine bell routine (DeMarco and Wüthrich, 1976) or using the convolution difference method (Campbell *et al*, 1973). Carr-Purcell A spectra were obtained as described by Campbell *et al* (1975).

Samples were prepared by lyophilizing the cytochromes c from a concentrated solution in 0.1M sodium phosphate buffer, pH 7.0, and repeating the lyophilisation a further two times following dissolution of the

lyophilisates in ^2H_2O. A third lyophilisation was performed following heating of the oxidized samples in ^2H_2O solution for 10 minutes at 55-60° in order to exchange the labile amide hydrogens for deuterium. The resulting lyophilisate was then dissolved in ^2H_2O to give a 2-5 mM solution of the cytochrome c. The solution was then transferred to an NMR tube, and flushed with argon. The reduced cytochromes c were obtained by addition of a small amount of solid sodium dithionite. The tubes were flushed once again with argon and sealed.

Solutions of the native protein, Hse^{65}-cytochrome c and Hse^{65},Leu^{74}-cytochrome c, thus prepared, remained in the reduced state for long periods and up to 97°C. The reduced form of Hse^{65},Leu^{67}-cytochrome c was less stable and appeared to be converted to the oxidized state (10-20%) after varying periods (5-15 h), depending on the temperature. Therefore, addition of sodium dithionite was required occasionally, resulting in a small decrease in p^{2}H of the samples. Furthermore, at 77° the protein was gradually lost by irreversible aggregation.

Redox potential measurements

Mid-point redox potentials at pH 7.0 ($E_{m,7}$) were determined with the ferricyanide-ferrocyanide redox couple. Solutions of native cytochrome c (5.6 μM) and Hse^{65},Leu^{67}-cytochrome c (7.2 μM) in 40 mM sodium phosphate buffer, pH 7.0, also containing 6.0 mM ferrocyanide, were titrated anaerobically with solutions of ferricyanide in the same buffer at 22°C. The amounts of reduced cytochromes c were determined spectrophotometrically by using a value of 20 $mM^{-1} \cdot cm^{-1}$ for the difference in extinction coefficient at the α-band wavelength. The mid-point potentials were calculated at half reduction of the cytochrome c, determined graphically from a plot of log ferro/ferri-cytochrome c against log ferro/ferri-cyanide, using a value of 0.423 V for the ferro-ferricyanide couple, calculated from the data of O'Reilly (1973).

Biological activity measurements

The reactivity of Hse^{65},Leu^{74}- and Hse^{65},Leu^{67}-cytochrome c with purified cytochrome c oxidase was determined using the ascorbate-TMPD system as described in Chapter VI. For details of the assay medium see legends to Figures 6 and 7.

The reaction between ferricytochrome c and ferrocytochrome c_1 was studied with a modified Durrum-Gibson stopped-flow apparatus as described by König *et al* (1980b). The reaction was studied in 250 mM potassium phosphate buffer, pH 7.0, containing 1% tween-20, at 10°C, and was followed by recording the spectral changes at 547.5 nm. *Pseudo* first order rate constants (k') were obtained for the reaction of cytochrome c_1 at a concentration of 1 μM with varying concentrations of cytochromes c (3-10 μM). The values of k' were plotted against the cytochrome c concentration. The slopes correspond to the second order rate constant k_1, with a standard deviation of approximately 10%.

In dit proefschrift wordt de ontwikkeling van een methode voor de semisynthese van cytochroom c (paardehart) analoga beschreven.

In het inleidende hoofdstuk worden de functie en structuur van cytochroom c kort beschreven. Tevens wordt daar de strategie gegeven die bij aanvang van het onderzoek werd opgesteld. Het schema berust op de waarneming van Corradin en Harbury (1974), dat in het 1:1 complex tussen de complementaire fragmenten 1-65 en 66-104 van cytochroom c een onverwacht snelle vorming van de Hse^{65}-Glu^{66} peptide band optreedt.

In Hoofdstuk II wordt aangetoond dat, bij gebruik van gezuiverde fragmenten, de aminolyse op het C-terminale homoserine lacton van het heem bevattende fragment 1-65 door de α-amino groep van Glu^{66}, nagenoeg kwantitatief verloopt. Het produkt, Hse^{65}-cytochroom c, onderscheidt zich alleen van het natieve eiwit door een substitutie op plaats 65 van methionine door homoserine. Aangetoond wordt dat deze verandering geen effect heeft op de electronen-transporterende functie van het eiwit.

In Hoofdstuk III wordt aangetoond dat de bescherming van de 19 ε-amino functies in cytochroom c met de methylsulfonylethyloxycarbonyl-(Msc)-groep snel en volledig verloopt bij reactie met een geringe overmaat aan Msc-ONSu in een dimethylformamide-water mengsel. Volledige afsplitsing van de beschermfunctie door een korte behandeling met base in dimethylformamide-methanol regenereert het natieve eiwit. Na splitsing van Msc-cytochroom c met behulp van cyanogeen bromide is het, selectief N^{ε}-beschermde, "natuurlijke" cytochroom c fragment 81-104 geisoleerd.

De synthese in oplossing van derivaten van de cytochroom c sequenties 66-80 en 66-79 wordt beschreven in Hoofdstuk IV. In het daartoe ontwikkelde syntheseschema worden de gewenste eindproducten opgebouwd uit drie fragmenten door twee opeenvolgende condensaties via een azide. Evenals in het "natuurlijke" fragment 81-104 is de Msc-groep de enige beschermfunctie in deze synthetische peptide derivaten.

De semisynthese van de cytochroom c sequentie 66-104 wordt beschreven in Hoofdstuk V. De beoogde koppeling tussen het synthetische $N^{\alpha 66}$, $N^{\varepsilon 72,73,79}$-tetra Msc-cytochroom c-(66-80)-pentadecapeptide azide en de

uit cytochroom c geïsoleerde N^{ε}-Msc-beschermde tetracosapeptide sequentie 81-104, bleek bemoeilijkt door een intramoleculaire nevenreactie van het azide. Uit modelexperimenten werd geconcludeerd dat de onbeschermde hydroxylfunctie van Thr^{78} daarvoor verantwoordelijk is.
Een tweede probleem vormde het associatiegedrag van het tetracosapeptide in media die water bevatten. De waarneming dat het "natuurlijke" fragment 81-104 goed oplost in dimethylsulfoxide, wanneer het vanuit een oplossing in trifluorazijnzuur wordt gedroogd, was daarom van belang. De azidekoppeling kon nu worden uitgevoerd bij concentraties, waarbij de bedoelde aminolyse concurrerend was met de nevenreactie.
De opbrengsten aan het cytochroom c-(66-104)-nonatriacontapeptide waren niettemin bescheiden (20-37%). Een verandering in de strategie had een gunstiger resultaat: het synthetische $N^{\alpha 66}$,$N^{\varepsilon 72,73,79}$-tetra Msc-cytochroom c-(66-79)-tetradecapeptide azide, waarin de positie van Thr^{78} ten opzichte van de azidefunctie was veranderd, vertoonde de genoemde nevenreactie niet. De acylering van het met methionine verlengde "natuurlijke" fragment 81-104 door dit tetradecapeptide azide, leverde dan ook - na afsplitsing van alle Msc-beschermfuncties - het semisynthetische cytochroom c-(66-104)-nonatriacontapeptide in goede opbrengsten (60-65%). Acylering van deze sequentie met het heem peptide 1-65 leverde Hse^{65}-cytochroom c (43%).

In Hoofdstuk V wordt tevens aangetoond dat dit, uit drie fragmenten opgebouwde Hse^{65}-cytochroom c eenzelfde biologische activiteit bezit als het natieve eiwit.

In de Hoofdstukken VI en VII wordt de ontwikkelde strategie toegepast in de synthese van analoga van cytochroom c, die substituties bevatten in de grotendeels invariante sequentie 66-80.

Het effect van het wegnemen van de positieve ladingen op de plaatsen 72, 73 en 79 in cytochroom c op de reactie met cytochroom c oxidase wordt onderzocht in Hoofdstuk VI. Het bleek dat Lys^{72} en Lys^{73} van even groot belang zijn bij de binding tussen de twee eiwitten, terwijl Lys^{79} een aanzienlijk geringere invloed daarop uitoefent.

De synthese van twee analoga, waarin de tyrosine residuen op de plaatsen 74 en 67 vervangen zijn door leucine, wordt beschreven in Hoofdstuk VII. Het bleek dat de functionele rol van beide aromatische residuen verschillend is. De substitutie van de aan het oppervlak van

het molecuul gelegen Tyr^{74} leidt tot een verlaagde stabiliteit van het geoxideerde cytochroom c, maar heeft geen invloed op de enzymatische eigenschappen. Uit de vervanging van Tyr^{67}, een diep in het molecuul gelegen residue, bleek daarentegen dat dit aminozuur van direct belang is voor de redox-eigenschappen van cytochroom c, o.a. tot uiting komend in een verhoogde reactiviteit met cytochroom c oxidase.
De interpretatie van de waargenomen verschillen was alleen mogelijk, nadat van beide analoga de structuur in oplossing was bestudeerd met behulp van hoge resolutie NMR spectroscopie en vergeleken met die van Hse^{65}-cytochroom c.

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Abbreviations

Ac	acetyl
Boc	t-butyloxycarbonyl
CM	carboxymethyl
DCC	N,N'-dicyclohexylcarbodiimide
DCHA	dicyclohexylamine
DCU	N,N'dicyclohexylurea
DEAE	diethylaminoethyl
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
EDPA	ethyldiisopropylamine
EtOAc	ethyl acetate
EtOH	ethyl alcohol
FDNB	1-fluoro-2,4-dinitrobenzene
HMPT	hexamethyl phosphoric triamide
HOBt	1-hydroxy benzotriazole
HONp	p-nitrophenol
HONSu	N-hydroxysuccinimide
Hse	homoserine
MeOH	methanol
Msc	2-(methylsulphonyl)ethyloxycarbonyl
OBu^t	t-butyloxy
OBzl	benzyloxy
OMe	methyloxy
ONp	p-nitrophenyloxy
OPfp	pentafluorophenyloxy
OTcp	2,4,5-trichlorophenyloxy
Pd-C	palladised (~10% Pd), activated charcoal
ppm	parts per million
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
TMPD	N,N,N',N'-tetramethyl-para-phenylenediamine
TosOH	p-toluenesulphonic acid

Trt trityl

Z benzyloxycarbonyl

TLC data

The following solvent systems were used to develop thin layer chromatograms:

A = chloroform-methanol (4:1)

B = chloroform-methanol-acetic acid (95:20:3)

C = n-butanol-acetic acid-water (4:1:1)

D = chloroform-methanol (9:1)

E = chloroform-methanol (1:1)

F = ethanol

G = ethyl acetate

H = n-butanol-pyridine-acetic acid-water (4:1:1:2)

I = n-butanol-pyridine-acetic acid-water (30:20:6:24)

J = ethyl acetate-methanol (7:3)

K = 2-propanol-25% ammonia-water (7:1:2)

L = chloroform-methanol (3:1)

M = ethyl acetate-methanol (4:1)

In order to remove pyridine, plates developed with solvent systems H and I were sprayed with concentrated ammonia solution and heated subsequently at ca 100°.

The following methods were used to detect components on thin layer plates:

- *the quenching of fluorescence* for detection of compounds containing aromatic groups: plates were exposed to UV-light (254 nm)
- *the TDM reagent* for detection of -NH-groups: plates were exposed to chlorine for approximately 10 seconds and the excess of chlorine was removed in a stream of air; spots were visualized by spraying the plates with a reagent obtained by mixing a solution of 4,4'-tetramethyldiaminodiphenyl-methane (TDM, 2.5 g) in acetic acid (10 ml) and water (50 ml) and a solution of potassium iodide (5 g) in water (100 ml; Von Arx, E., Faupel, M. and Brugger, M. (1976) J. Chromatogr. *120*, 224-228)
- *the ninhydrine reagent* for detection of free amino groups: plates were sprayed with a solution of ninhydrine (240 mg) in butanol-1 (400 ml)

and acetic acid (16 ml), followed by heating the plates in a stream of hot air (~80°) for 5-10 minutes

-*the Pauly reagent* for detection of histidine containing compounds: plates were sprayed with a freshly prepared solution of diazotized sulphanilic acid (approximately 50 mg of the moist compound) in 5% Na_2CO_3 (10 ml)

-*the Barton reagent* for the detection of hydrazides and tyrosine containing compounds: plates were sprayed with a reagent obtained by mixing, just before use, equal volumes of a solution of $FeCl_3$ (30 g) in water (170 ml) and a solution of $K_3Fe(CN)_6$ (2 g) in water (200 ml)

-*the Ehrlich reagent* for the detection of tryptophan containing compounds: plates were sprayed with a solution of 4-dimethylaminobenzaldehyde (1.0 g) in conc HCl (25 ml) and methanol (75 ml), followed by heating the plates in a stream of hot air (~80°).

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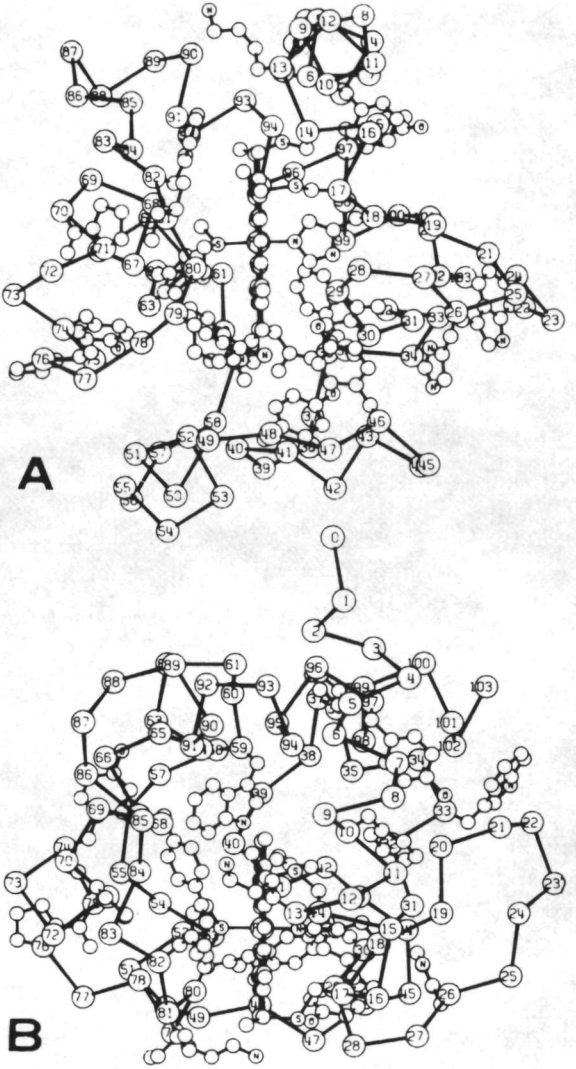
Na het behalen van het diploma H.B.S.-B diploma aan het Pius X College te Beverwijk in 1966, begon hij in 1967 met de studie in de Scheikunde aan de Katholieke Universiteit te Nijmegen.
Het kandidaatsexamen (S2) werd in september 1970 afgelegd.

De doctoraalstudie omvatte het hoofdvak Biochemie (Prof.dr S.L. Bonting en Prof.dr H. Bloemendal) en de bijvakken Organische Chemie (prof.dr R.J.F. Nivard en dr G.I. Tesser) en Exobiologie (Prof.dr A.W. Schwartz en dr G.J.F. Chittenden). Het doctoraalexamen werd afgelegd in februari 1975.

Van 1 maart 1975 tot 1 mei 1979 was hij als wetenschappelijk medewerker - in dienst van de Stichting Scheikundig Onderzoek Nederland (SON) - werkzaam op de Afdeling Organische Chemie van de Katholieke Universiteit te Nijmegen. Hier werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof.dr R.J.F. Nivard en dr G.I. Tesser.

Hij is gehuwd en heeft drie dochters.



α-carbon drawing of the backbone and heme group of tuna ferrocytochrome c (2 Å resolution); the side chains connected to the heme and those with flat rings are shown (reproduced with permission from Takano *et al* (1977)).

A. Front view; B. Top view.

I

De door Nix en Warme (1979) beschreven procedure voor de semisynthese van Hse^{65}-cytochroom c, waarin wordt afgezien van een doelmatige zuivering en karakterisering van het product, is ongeschikt om bij te dragen aan het inzicht in de structuur-functierelatie van cytochroom c.

P.T. Nix en P.K. Warme (1979) Biochim. Biophys. Acta 578, 413-427.
A.K. Koul, G.F. Wasserman en P.K. Warme (1979) Biochem. Biophys. Res. Comm. 89, 1253-1259.
G.F. Wasserman, P.T. Nix, A.K. Koul en P.K. Warme (1980) Biochim. Biophys. Acta 623, 457-460.

II

De onlangs door Ambler *et al* (1981) gecorrigeerde aminozuurvolgorde van cytochroom c_{550} van *Paracoccus denitrificans* toont aan dat het verwerpen van het Dickerson-Winfield mechanisme voor de reductie van cytochroom c door Dickerson en Timkovich in 1975 voorbarig was.

R.P. Ambler, T.E. Meyer, M.D. Kamen, S.A. Schichman en L. Sawyer (1981) J. Mol. Biol. 147, 351-356.
R.E. Dickerson en R. Timkovich in: "The Enzymes" (Boyer, P., ed.) 3rd edn., Vol. 11, pp. 397-547. Academic Press, New York, 1975.

III

Pande en Meyer (1980) geven geen verklaring voor het verschil in reactiviteit van de twee guanidinofunkties met 2,3-butaandion in respectievelijk ferri- en ferrocytochroom c. Op grond van hun experimentele gegevens kan het verschil aan een licht-afhankelijke nevenreactie worden toegeschreven.

J. Pande en V.P. Meyer (1980) J. Biol. Chem. 255, 11094-11097.
H. Fliss en T. Viswanatha (1979) Can. J. Biochem. 57, 1267-1272.

IV

Primitieve eiwitten bestaan niet.

R.F. Doolittle in: "The Proteins" (Neurath, H., Hill, R.L., eds.) 3rd edn., Vol. IV, pp 1-118. Academic Press, New York, 1979.

V

Bij de toewijzing van de configuratie van het sulfoxide S-atoom in een 5-ring sultine met behulp van NMR-spectroscopie maken Sharma *et al* (1976) ten onrechte gebruik van het voor een 6-ring sultine waargenomen deshielding effect.

Sharma, N.K., Reinach-Hirtzbach de, F. and Durst, T. (1976) Can. J. Chem. 54, 3012-3025.

VI

De bewering van Chou en Ganem (1980) dat zij als eersten de totaalsynthese van het iso-epoxydon, een intermediair in de biosynthese van patuline, hebben uitgevoerd, is onjuist.

D.T. Chou en B. Ganem (1980) J. Am. Chem. Soc. 102, 7987-7988.
A. Ichihara, R. Kimura, K. Oda en S. Sakamura (1976) Tetrahedron Letters, 4741-4744.

VII

De verklaring van Huisgen en Schug (1976) voor de afnemende reactiviteit van 1,1-di-, tri- en tetracyanoethenen in de 2+2 cycloadditie met enolethers, ten gevolge van sterische hindering en verlies van conjugatie, berust op een onderschatting van de invloed van de betrokken grensorbitalen op de reactiviteit.

R. Huisgen en R. Schug (1976) J. Am. Chem. Soc. 98, 7819-7821.
R. Huisgen (1981) Pure Appl. Chem. 53, 171-187.

VIII

Gezien het feit dat glutathion een lichaamsbeschermende functie uitoefent, roept het enige verbazing op dat broomsulfoftaleïne (BSP) nog wordt gebruikt om leverfuncties van patiënten te testen.

S.J. Hoorntje in: "Lever en galwegen - diagnostiek en therapie" (Gips, C.H. en Wilson, J.H.P., eds.) pp 27-33, Bohn, Scheltema en Holkema, Utrecht, 1979.
L.F. Chasseaud in: "Glutathione: Metabolism and Function" (Arias, I.M. en Jakoby, W.B., eds.) pp 77-114. Raven Press, New York, 1976.

IX

De negatieve instelling van veel journalisten bij de voorlichting over chemie kan als een puberteitsverschijnsel worden beschouwd in een voortschrijdend democratiseringsproces.

Nijmegen, 15 juni 1981 P.J. Boon